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Physiological Zoölogy

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ACTION OF IONS ON THE HEART OF THE OYSTER (OSTREA VIRGINICA)[1]

(Ten figures)

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Department of Zoölogy, Johns Hopkins University

RINGER (1882) showed that sodium, potassium, and calcium salts are normally present in definite proportions in the blood of vertebrates, and are necessary for the proper functioning of the tissues. He studied the effect of these salts mainly on the heart of the frog, and found that excess of sodium or potassium in the fluid perfusing the heart causes cessation of beating in a relaxed condition, i.e., arrest in diastole, while excess of calcium causes cessation of beating in a contracted condition, i.e., arrest in systole; also that deficiency of sodium or potassium has the same effect as excess of calcium, while deficiency of calcium has the same effect as excess of either of the other two.

Experiments on the effects of salts have been performed on the hearts of a large number of other species of animals by other investigators. The results obtained on the hearts of all vertebrates are in general agreement with those obtained by Ringer on the heart of the frog, but the results obtained on the hearts of a considerable number of invertebrates often appear to be the reverse in kind of those obtained with vertebrate hearts.

The results obtained with the hearts of invertebrates show that excess of NaCl causes arrest in systole in *Limulus* (Carlson, 1906), *Maia* and *Homarus* (Hogben, 1925), *Cambarus clarkii* (Lindeman, 1928), and *Helix pomatia* (Cardot and Jullien, 1930); that excess of KCl causes arrest in systole in *Aplysia* (Heymans, 1923) and *Helix* (Cardot and Jullien, 1930); and that excess of $CaCl_2$ causes arrest in diastole in *Aplysia* (Heymans, 1923), *Helix* (Cardot, 1921, and Hogben, 1925), *Ostrea circumpicta* Pils. (Takatsuki, 1927), and *Maia* (Hogben, 1925); but that excess of either potassium or calcium causes arrest in systole in *Pecten* (Mines, 1912) and *Anodon* (Koch, 1916).

[1] I wish to express my appreciation to Professor S. O. Mast, under whose direction this problem was carried out, for his many suggestions, especially in reference to the preparation of this paper for publication; and to Professor Ivon R. Taylor, Brown University, for his help and supervision of all phases of the work.

I am also greatly indebted to the Biological Laboratory, Cold Spring Harbor, L.I., N.Y., for a grant which covered nearly all the expenses incurred, and for providing excellent laboratory facilities, apparatus, etc.

A thorough investigation of the action of salts on one of the invertebrates exhibiting anomalous effects is obviously necessary in attempting to explain the differences observed. The heart of the oyster exhibits anomalous effects to a marked degree, and, since it is readily obtained and kept, it was chosen for the following experiments.

METHODS

Large oysters of uniform size were collected in Oyster Bay, Long Island Sound, during July and kept in a live box in Cold Spring Harbor until used. All experiments were made during July, August, and September.

The heart of the oyster is located in the pericardial cavity directly behind the adductor muscle. The rectum runs adjacent to the tip of the ventricle, and is closely attached to the arteries which leave the ventricle and run anteriorly and posteriorly, respectively, along the gut, but it does not penetrate the substance of the heart as is true of some lamellibranches. The walls of the auricles are thin, and they are composed of unstriated muscle fibers which are loosely interwoven and present a meshlike appearance.

The excised heart of the oyster, like that of other marine invertebrates, beats normally for from several hours to several days if it is perfused with sea water or van't Hoff solution. However, since the oysters used were collected in brackish waters, and freezing-point determinations of the blood of these oysters showed it to be hypotonic with van't Hoff solution, this solution was diluted until its conductivity equaled that of the blood of the oyster. The concentration of the salts in the diluted van't Hoff solution follows: 0.36 molar NaCl, 0.028 molar $MgCl_2$, 0.014 molar $MgSO_4$, 0.008 molar KCl, 0.011 molar $CaCl_2$.

The solutions used to ascertain the effect of different ions on the heart consisted either of solutions of single salts or of diluted van't Hoff solution in which the relative concentrations of the salts had been changed. Dextrose was added to the solutions of single salts, if necessary, to make them isosmotic with the blood of the oyster. The solutions were unbuffered, but acid or base was added so that, immediately before using, the H-ion activity was the same as that of the blood of the oyster. The solutions were protected from access of carbon dioxide by means of soda-lime tubes, and tests showed no appreciable changes in the H-ion activity during the experiments. Mallinckrodt's or Baker's Analyzed salts were used. Solutions of deliquescent chlorides were standardized against silver nitrate according to the Volhard-Drechsel method. A triple-tandem pyrex glass still containing (*a*) $KMnO_4$, (*b*) $Ba(OH)_2$, and (*c*) distilled water (Mast, 1928) was used in preparing the distilled water for the solutions. The solutions were prepared and mixed immediately before using.

The hearts used were prepared for perfusion as follows: The shell was opened by cutting the adductor muscle, and the heart canulated by inserting a small glass canula, through a cut in one of the auricles, into the ventricle (Fig. 1). It was then tied in place by a ligature at the auriculo-ventricular junction, after which the heart was removed and the canula connected to the outlet of the stopcock in the perfusion chamber (Fig. 1). Tracings of the contractions of the heart were made on a long-paper kymograph by means of a writing lever.

In testing the action on the heart of solutions, they were put into bottles *A2*, *A3*, and *A4*, and van't Hoff solution into *A1* (Fig. 2). The stopcocks *S1–S4* (Fig. 2) were then adjusted until the liquid rose to the same level in the manometers *M1–M4*, so as to make the flowing pressure during perfusion the same for all solutions. The perfusion fluid was

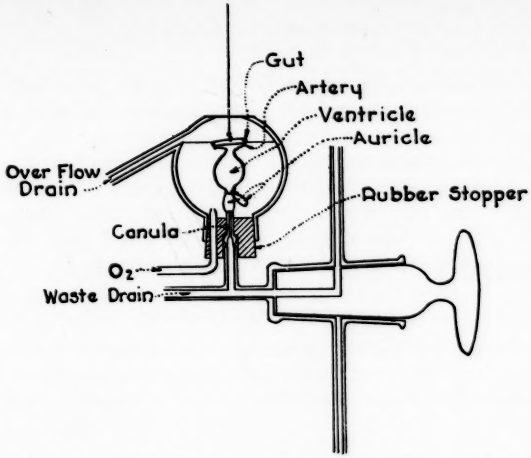

FIG. 1.—Sketch showing method of perfusion of the heart

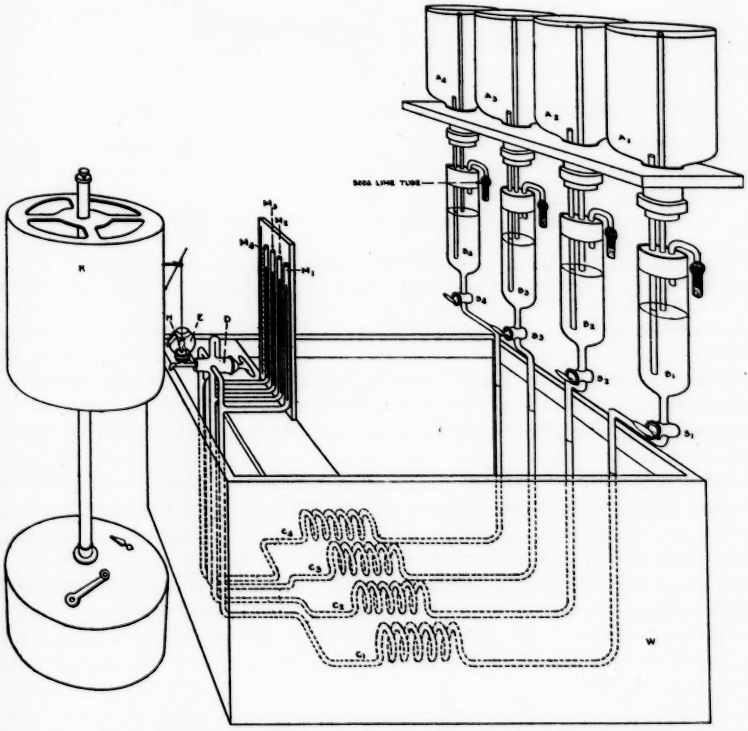

FIG. 2.—Sketch showing arrangement of apparatus used in perfusing the heart of the oyster. *A1–A4*, reservoirs containing perfusion fluids; *B1–B4*, constant-level tubes; *D*, four-way stopcock; *H*, heart; *K*, kymograph; *M1–M4*, manometers; *S1–S4*, stopcocks for regulating the flowing pressures; *V*, perfusion chamber; *W*, constant-temperature water bath.

brought to 18° C. by passing it through thin-walled pyrex-glass coils, *C1–C4* (Fig. 2) in a thermostatically controlled water bath. Tests showed that the temperature of the fluid in the heart chamber did not vary by more than 0°.5 C.

The observations concerning the effects of salts were made as follows: The excised heart was perfused with van't Hoff solution until the beat had become well established; then it was perfused with the solution to be tested until the character of the response again became steady; after which it was again perfused with van't Hoff solution. If the heart did not completely recover, the results were discarded.

SODIUM CHLORIDE

The action of sodium chloride on the hearts of several other mollusks closely related to the oyster, *O. virginica*, has been studied by other investigators and extremely diverse results were obtained. Heymans (1923) found that solutions of NaCl cause arrest in

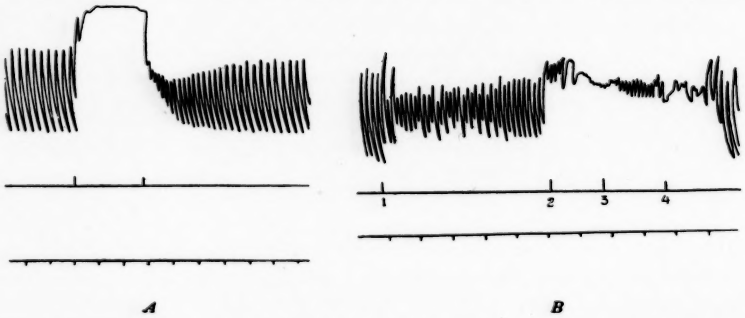

FIG. 3.—Graphs showing the effect of solutions containing different concentrations of NaCl, made isotonic with the blood of the oyster by the addition of dextrose. *A*, 0.418 molar; *B:* (1) van't Hoff solution changed to 0.104 molar NaCl, (2) 0.104 molar NaCl changed to 0.418 molar NaCl, (3) 0.418 molar NaCl changed to 0.104 molar NaCl, (4) 0.104 molar NaCl changed to van't Hoff solution.

systole in the heart of the parapod, *Aplysia;* Takatsuki (1927) maintained that it exerts little or no effect on the heart of the oyster, *O. circumpicta* Pils.; and Cardot and Jullien (1930) concluded that sodium chloride specifically inhibits contraction in the heart of the land snail, *H. pomatia*.

It has been maintained that the results obtained from perfusion with pure solutions of NaCl are due to the absence of the other necessary salts, and consequently do not give a true indication of the action of sodium. However, if various concentrations are used, the effect due to the sodium ion should become apparent.

Solutions containing 0.418 molar, 0.209 molar, and 0.04 molar NaCl and sufficient dextrose to make them isotonic with the blood of the oyster were tested on a considerable number of hearts. There was but little variation in the results obtained. Those of a typical experiment on one heart are presented in Figure 3, and the average for all the experiments in Table 1.

Figure 3 and Table 1 show that with 0.418 molar and 0.209 molar NaCl the heart immediately contracted and remained in this condition without beating until it was again perfused with van't Hoff solution; and that the greater contraction was obtained

with the stronger solutions. Figure 3*b* and Table 1 show that with 0.104 molar NaCl the heart did not stop beating, but that the extent of relaxation decreased; and that, if the heart is perfused with 0.104 molar NaCl after it has stopped beating in 0.418 molar NaCl, it resumes beating. This demonstrates clearly that the stoppage of the heart with 0.418 molar NaCl is due to action of the sodium ion and not to lack of the other ions.

TABLE 1

EFFECT ON THE BEAT OF THE HEART OF THE OYSTER OF SOLUTIONS OF DIFFERENT CONCENTRATIONS OF NaCl, MADE ISOTONIC WITH THE BLOOD OF THE OYSTER BY THE ADDITION OF DEXTROSE

Concentration of NaCl	Percentage Change in Frequency	Percentage Change in Amplitude	Change in Extent of Relaxation (Mm.)
0.418 molar	−100	−100	−7.1
0.209	−100	−100	−3.5
0.104	0	− 27	−1.8

It can be concluded that solutions of sodium chloride inhibit relaxation in the heart of the oyster and, in high concentrations, cause arrest with augmentation of normal contraction. These results are obviously not in accord with the findings of Ringer (1882), Lussana (1911), Pond (1920), and others with vertebrate hearts, or with the conclusions of Cardot and Jullien (1930) that sodium specifically inhibits contraction in the heart of *Helix;* but they are in accord with those of Carlson (1906) on the heart of *Limulus*, and Hogben (1926) on those of *Homarus* and *Maia*.

POTASSIUM CHLORIDE

PURE SOLUTIONS OF KCl IN DIFFERENT CONCENTRATIONS

Experiments similar to those just described were performed on a considerable number of hearts with solutions containing the following concentrations of KCl: 0.418 molar, 0.209 molar, 0.104 molar, 0.04 molar, and 0.008 molar, and sufficient dextrose to make them isotonic with the blood of the oyster.

Figure 4 and Table 2 show that KCl in concentrations from 0.418 molar to 0.04 molar causes the heart to stop in a contracted state which is greater than that of normal systole, but that the extent of contraction varies directly with the concentration of KCl; and that 0.008 molar KCl does not cause the heart to stop immediately but causes decrease in frequency and amplitude and a tendency to sustained contraction.

These results are somewhat similar to those obtained with solutions of NaCl. The relation between concentration of salt and extent of contraction for NaCl and KCl is presented in Figure 5. This figure shows that solutions of KCl produce greater contraction than solutions of NaCl of the same concentration, and that greater concentrations of NaCl are required to stop the heart than of KCl.

EXCESS OF KCl IN VAN'T HOFF SOLUTION

Experiments were performed with van't Hoff solutions with the concentration of KCl increased two, four, and six times, respectively (0.016 molar, 0.032 molar, and 0.048

molar), but with the concentrations of the other salts unchanged. The results of some of these experiments are given in Figure 6*a* and Table 2.

Reference to Figure 6*a* and Table 2 shows that, if the heart is perfused with van't Hoff solution containing excess of KCl, the frequency of beat increases and the extent of relaxation decreases. Comparison of the results obtained with different concentrations of KCl presented in Table 2 shows that, if the concentration of the salt is increased, the extent of relaxation decreases until with the concentration of KCl increased six times the heart stops in systole.

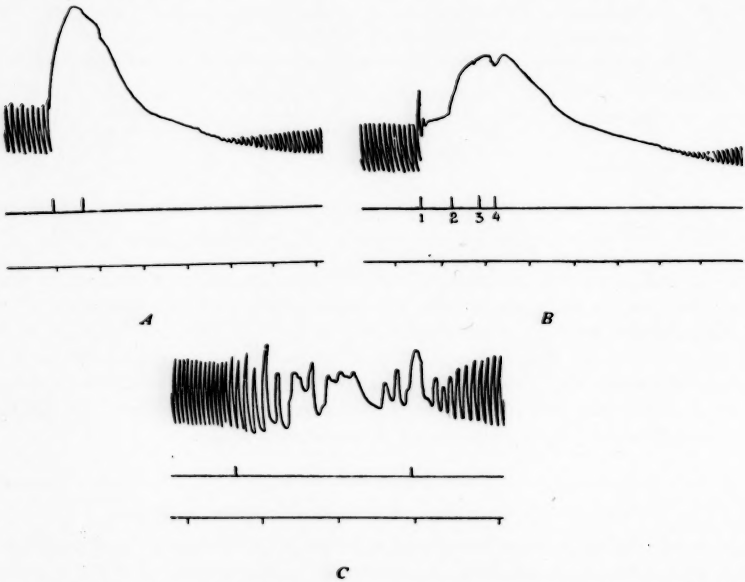

FIG. 4.—Graphs showing the effect of solutions of different concentrations of KCl, made isotonic with the blood of the oyster by the addition of dextrose. *A*, 0.418 molar KCl, *B:* (1) van't Hoff solution changed to 0.04 molar KCl, (2) 0.04 molar KCl changed to 0.209 molar KCl, (3) 0.209 molar KCl changed to 0.418 molar KCl, (4) 0.418 molar KCl changed to van't Hoff solution; *C*, 0.008 molar KCl.

DEFICIENCY OF KCl IN VAN'T HOFF SOLUTION

Experiments were performed in which the concentration of KCl in van't Hoff solution was decreased from 0.008 molar to 0.0045 molar, 0.003 molar, 0.0015 molar, 0.0007 molar, and 0.0 molar, respectively. The results of these experiments are presented in Figure 6*b* and *c* and Table 2.

These results show that decrease in concentration of KCl in van't Hoff solution to 0.003 molar causes increase in frequency—and inhibition of relaxation—but no change in extent of contraction at systole; that decrease to 0.0015 molar causes decrease in frequency and extent of contraction as well as inhibition of relaxation; and that further decrease causes arrest in systole.

It has been frequently assumed in the literature that, if excess of a salt produces a

certain effect, deficiency of it should produce the opposite effect. This obviously does not obtain with the oyster heart. The results obtained indicate that there is an optimum concentration of KCl, and that either increase or decrease in this concentration results in inhibition of relaxation. It is of interest to observe that, whereas excess of KCl causes arrest in diastole in vertebrate hearts and arrest in systole in the oyster heart, deficiency of KCl has the same effect on both, i.e., inhibition of relaxation.

TABLE 2

EFFECT ON THE BEAT OF THE HEART OF THE OYSTER OF SOLUTIONS OF DIFFERENT CONCENTRATIONS OF KCl, MADE ISOTONIC WITH THE BLOOD OF THE OYSTER BY THE ADDITION OF DEXTROSE, AND OF VAN'T HOFF SOLUTIONS WITH DIFFERENT DEGREES OF EXCESS AND DEFICIENCY OF KCl

Concentration of KCl	Percentage Change in Frequency	Percentage Change in Amplitude	Change in Extent of Relaxation (Mm.)
	KCl in Dextrose Solutions		
0.418 molar	−100	−100	−8.5
0.209	−100	−100	−6.8
0.104	−100	−100	−4.6
0.04	−100	−100	−4.1
0.008	− 50	− 53	−0.8
	KCl in van't Hoff Solution (Excess)		
0.016	+ 80	− 82	−3.3
0.032	+ 25	− 99	−4.6
0.048	−100	−100	−4.9
	KCl in van't Hoff Solution (Deficiency)		
0.0045	+ 9	− 47	−1.6
0.003	+ 33	− 73	−2.1
0.0015	− 6	− 83	−1.6
0.0007	−100	−100	−2.8
0.0	−100	−100	−3.8

CALCIUM CHLORIDE

PURE SOLUTIONS OF $CaCl_2$ IN DIFFERENT CONCENTRATIONS

Experiments were made with four different concentrations of $CaCl_2$ (0.418 molar, 0.209 molar, 0.104 molar, and 0.04 molar) on a considerable number of hearts. The results are presented in Figure 7 and Table 3. Figure 7*a* and Table 3 show that 0.418 molar $CaCl_2$ causes the heart to stop beating immediately in a state of relaxation much greater than it is in normal diastole.

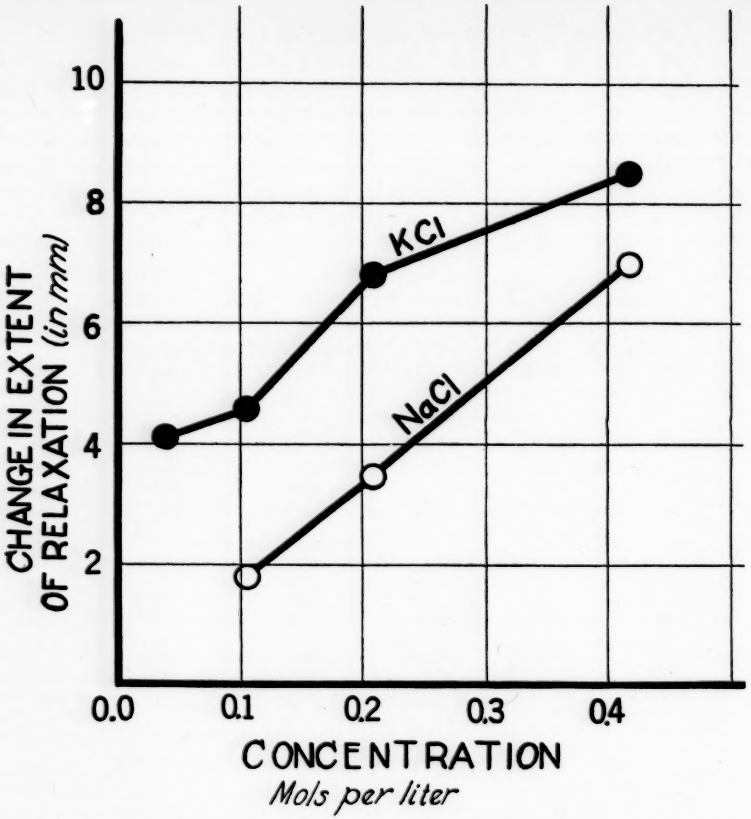

FIG. 5.—Graphs showing the relation between concentration of salt and the change in extent of relaxation. *Abscissas*, concentration of salt; *ordinates*, change in extent of relaxation.

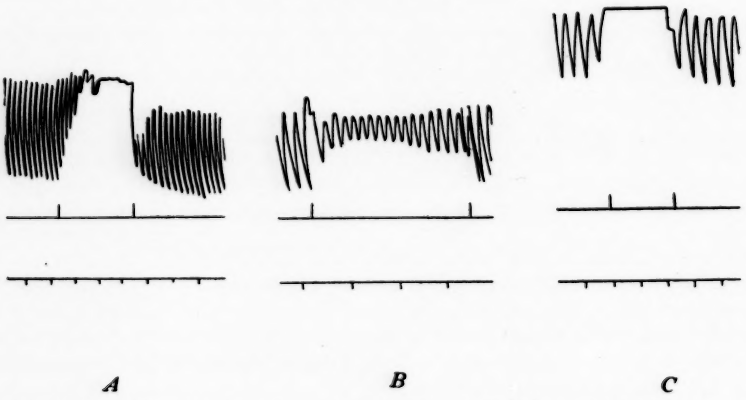

FIG. 6.—Graphs showing the effect of excess and deficiency of KCl in van't Hoff solution. *A*, van't Hoff solution with KCl increased to 0.045 molar; *B*, van't Hoff solution with KCl decreased to 0.0045 molar; *C*, van't Hoff solution with no KCl.

At the second signal shown on graph *A* (Fig. 7) the heart was stimulated with an induction shock. The graph shows that this was followed by a single normal contraction. This demonstrates that the heart is still capable of contracting, indicating that the diastolic arrest was caused by interruption of automatic stimulation. Graph *A* also shows that when a heart, which has stopped in diastole from perfusion with a strong solution of $CaCl_2$, is again perfused with van't Hoff solution it immediately contracts very strongly, and then slowly relaxes until normal beating is resumed.

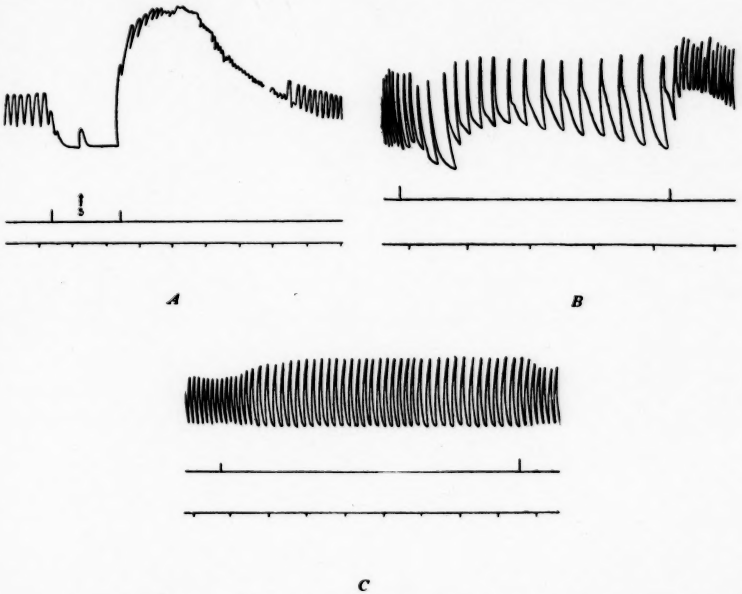

FIG. 7.—Graphs showing the effect of solutions of different concentrations of $CaCl_2$, made isotonic with the blood of the oyster by the addition of dextrose, and of van't Hoff solution with excess of $CaCl_2$. *A*, 0.418 molar; *B*, 0.104 molar; *C*, van't Hoff solution with $CaCl_2$ increased to 0.17 molar.

Table 3 shows that 0.209 molar $CaCl_2$ also causes the heart to stop immediately in diastole, but that the extent of relaxation and the extent of contraction preceding recovery on perfusion with van't Hoff solution are less than they are with 0.418 molar $CaCl_2$. Graph *B* (Fig. 7) and Table 3 show that with concentrations of $CaCl_2$ below 0.104 molar the heart continues beating, but with decrease in frequency; and that with decrease in concentration of $CaCl_2$ the frequency of beat increases and the amplitude of contraction decreases.

The foregoing results indicate that calcium acts in two ways on the heart of the oyster, i.e., that it depresses excitation and augments contraction. The decrease in frequency accompanying increase in concentration and the diastolic arrest which occurs in concentrated solutions are obviously due to inhibition of the mechanism associated with excitation rather than to an effect on the contraction itself, since under these conditions the heart still responds to artificial stimulation.

The increase in amplitude of contraction accompanying increase in concentration of $CaCl_2$ demonstrates the augmenting action of this salt on contraction, and it is believed that the abnormally strong contraction preceding recovery in van't Hoff solution of a heart stopped in diastole by means of perfusion with a strong solution of $CaCl_2$ also demonstrates this; i.e., the $CaCl_2$ that accumulates in the tissues during perfusion with the $CaCl_2$ solution causes augmentation of contraction on the resumption of automatic excitation in van't Hoff solution.

TABLE 3

EFFECT ON THE BEAT OF THE HEART OF THE OYSTER OF SOLUTIONS OF DIFFERENT CONCENTRATIONS OF $CaCl_2$, MADE ISOTONIC WITH THE BLOOD OF THE OYSTER BY THE ADDITION OF DEXTROSE, AND OF VAN'T HOFF SOLUTIONS WITH DIFFERENT DEGREES OF EXCESS AND DEFICIENCY OF $CaCl_2$

Concentration of $CaCl_2$	Percentage Change in Frequency	Percentage Change in Amplitude	Change in Extent of Relaxation (Mm.)
	$CaCl_2$ in Dextrose Solutions		
0.418 molar	−100	−100	+1.1
0.209	−100	−100	+0.6
0.104	− 71	+ 10	0.0
0.04	− 50	+ 3.6	0.0
	$CaCl_2$ in van't Hoff Solution (Excess)		
0.022	−15	+13	0.0
0.044	−25	+22	0.0
0.088	−25	+30	+0.1
0.17	−35	+48	+0.1
	$CaCl_2$ in van't Hoff Solution (Deficiency)		
0.003	+ 25	− 77	−3.0
0.005	+ 98	− 32	−0.8
0.008	+ 66	− 1.2	−0.2
0.0	−100	−100	0.0

EXCESS OF $CaCl_2$ IN VAN'T HOFF SOLUTION

Experiments were performed with van't Hoff solutions in which the concentration of $CaCl_2$ was increased, but without any change in the concentration of the other salts. Solutions in which the concentration of $CaCl_2$ was increased two, four, eight, and sixteen times (0.022 molar, 0.044 molar, 0.088 molar, and 0.17 molar, respectively) were used. The results for one experiment for one concentration are presented in graph *C* (Fig. 7), and the averages for all the concentrations used are presented in Table 3.

Graph *C* (Fig. 7) and Table 3 show that van't Hoff solutions containing excess of

$CaCl_2$ cause decrease in frequency of beat, increase in amplitude of contraction, and, in high concentration, increase in extent of relaxation. Figure 8 shows that the decrease in frequency and increase in amplitude of contraction vary directly with increase in $CaCl_2$ concentration.

These results obviously support the conclusion reached in the experiment on the effect of solutions of $CaCl_2$, namely, that calcium has a depressing effect on excitation as well as an augmenting effect on contraction.

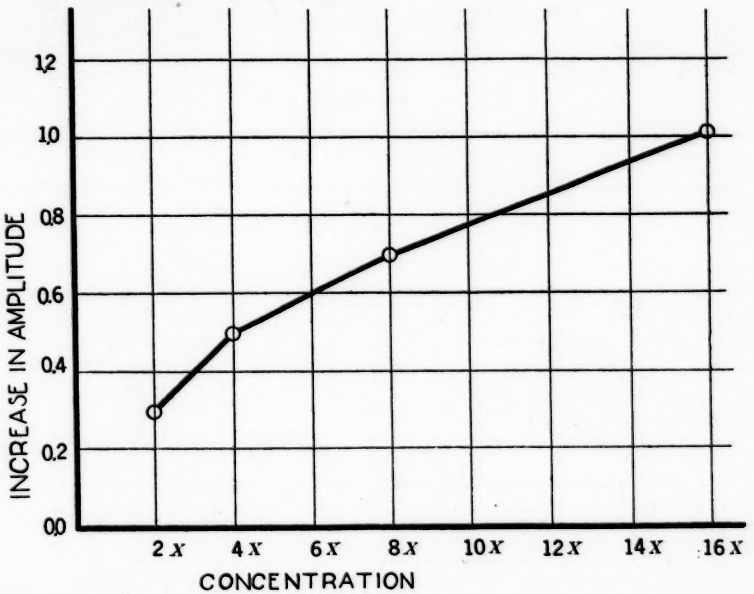

FIG. 8.—Graphs showing the relation between increase in concentration of $CaCl_2$ in van't Hoff solution and the change in amplitude of contraction. *Abscissas*, concentration of $CaCl_2$ (*X*, 0.011 molar $CaCl_2$, i.e., the concentration of $CaCl_2$ in van't Hoff solution); *ordinates*, change in amplitude of contraction.

DEFICIENCY OF $CaCl_2$ IN VAN'T HOFF SOLUTION

Experiments were performed in which the concentration of $CaCl_2$ in van't Hoff solution was decreased to 0.008 molar, 0.005 molar, 0.003 molar, and 0.0 molar, respectively. Figure 9*a* contains the results obtained in an experiment in which the heart was perfused successively with van't Hoff solutions containing 0.008 molar, 0.005 molar, and 0.003 molar $CaCl_2$, respectively. The results are essentially the same as those obtained in experiments in which each concentration was tested singly. Figure 9*b* shows the effect of complete absence of $CaCl_2$. Table 3 contains the averages of the results obtained with each of these solutions on a number of hearts.

Graph *A* (Fig. 9) and Table 3 show that perfusion with van't Hoff solution, with $CaCl_2$ decreased to 0.005 molar, causes inhibition of relaxation and increase in frequency, but does not affect the amplitude of contraction; that perfusion with van't Hoff solution

with the concentration of $CaCl_2$ lower than 0.005 molar causes decrease in extent of contraction as well as inhibition of relaxation and increase in frequency; and that perfusion with van't Hoff solution containing no $CaCl_2$ causes increase in length of the systolic period with gradual decrease in amplitude of contraction until the heart stops in diastole.

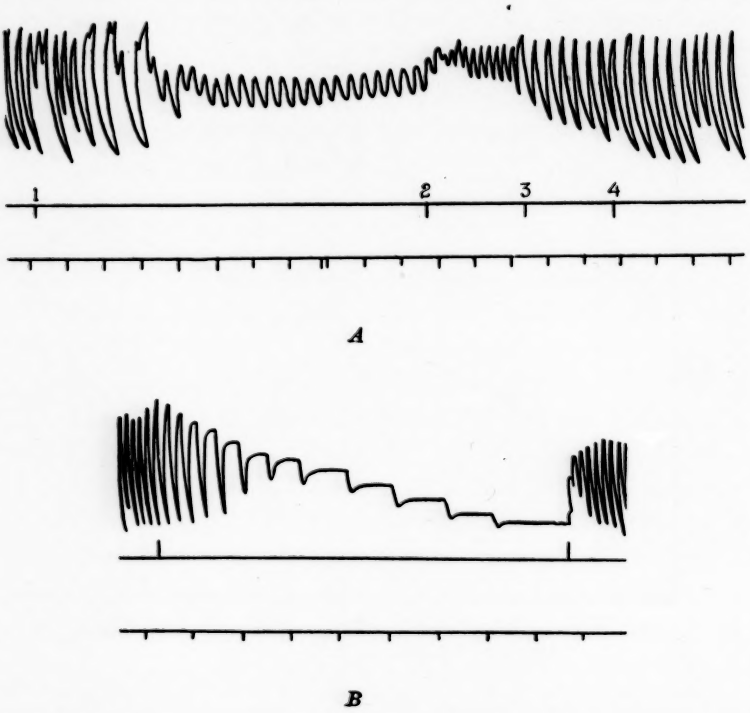

FIG. 9.—Graphs showing the effect of van't Hoff solution with deficiency of $CaCl_2$. *A:* (1) van't Hoff solution containing 0.011 molar $CaCl_2$ changed to van't Hoff solution containing 0.003 molar $CaCl_2$, (2) van't Hoff solution containing 0.003 molar $CaCl_2$ changed to 0.005 molar $CaCl_2$, (3) van't Hoff solution containing 0.005 molar $CaCl_2$ changed to van't Hoff solution containing 0.008 molar $CaCl_2$, (4) van't Hoff solution containing 0.008 molar $CaCl_2$ changed to van't Hoff solution containing 0.011 molar $CaCl_2$; *B*, effect of van't Hoff solution containing no $CaCl_2$.

It seems probable that the inhibition of relaxation and increase in frequency caused by van't Hoff solution with decreased concentration of $CaCl_2$ are due to diminished antagonism of the action of the other ions in van't Hoff solution. The decrease in the extent of contraction obtaining with $CaCl_2$ concentrations below 0.008 molar and the delayed diastolic arrest with complete lack of $CaCl_2$ indicate that, like vertebrate hearts, the oyster heart requires calcium for the functioning of the contractile mechanism.

MAGNESIUM CHLORIDE

PURE SOLUTIONS OF $MgCl_2$ IN DIFFERENT CONCENTRATIONS

Experiments were performed using two concentrations of $MgCl_2$ (0.418 molar and 0.209 molar). The results are presented in Figure 10 and Table 4. These show that solutions of $MgCl_2$ in both these concentrations cause the heart to stop in a greatly relaxed state, but that the extent of relaxation is less with the lower concentration.

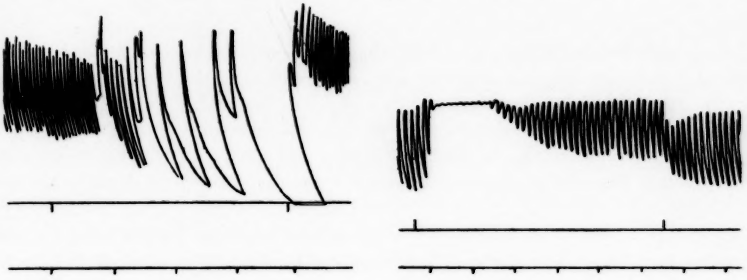

FIG. 10.—Graphs showing the effect of solutions containing different concentrations of $MgCl_2$, made isotonic with the blood of the oyster by the addition of dextrose, and of van't Hoff solution lacking magnesium salts. *A*, 0.418 molar $MgCl_2$; *B*, van't Hoff solution lacking magnesium salts.

TABLE 4

EFFECT ON THE BEAT OF THE HEART OF THE OYSTER OF SOLUTIONS OF DIFFERENT CONCENTRATIONS OF $MgCl_2$, MADE ISOTONIC WITH BLOOD OF THE OYSTER BY THE ADDITION OF DEXTROSE, AND OF VAN'T HOFF SOLUTION WITH DEFICIENCY OF MAGNESIUM SALTS

Concentration of $MgCl_2$	Percentage Change in Frequency	Percentage Change in Amplitude	Change in Extent of Relaxation (Mm.)
	$MgCl_2$ in Dextrose Solutions		
0.418 molar	−100	−100	+5.0
0.209	−100	−100	+4.5
	$MgCl_2$ in van't Hoff Solution (Deficiency)		
0.0	+20	−9.5	−0.3

These results resemble those obtained with solutions of $CaCl_2$. The figure shows, however, that the abnormally strong contraction observed immediately after the

calcium solution was replaced by van't Hoff solution, did not occur when the magnesium solution was replaced by van't Hoff solution. The fact that contraction preceding recovery with van't Hoff solution did not occur indicates that magnesium does not have an augmenting effect on contraction like that of calcium.

DEFICIENCY OF MAGNESIUM IN VAN'T HOFF SOLUTION

Experiments were performed with solutions containing different concentrations of magnesium salts in van't Hoff solution. The concentration of the other ions was not changed. No definite changes were observed in the beat of the heart except with magnesium entirely lacking. The results obtained with van't Hoff solution without magnesium are presented in Figure 10 and Table 4. These results show that lack of magnesium in van't Hoff solution causes inhibition of relaxation and increase in frequency.

Comparison of the results concerning magnesium with those concerning calcium indicates that these ions have similar effects on excitation but that magnesium is not necessary for the functioning of the contractile mechanism, while calcium is. The increase in frequency and the inhibition of relaxation produced by lack of magnesium in van't Hoff solution indicate that magnesium, like calcium, antagonizes sodium and potassium.

DISCUSSION

The foregoing results show that the effects of NaCl, KCl, $CaCl_2$, and $MgCl_2$ on the heart of the oyster are not in complete accord with the effects of these salts on the hearts of vertebrates. The chief differences are that excess of KCl causes inhibition of relaxation in the heart of the oyster and inhibition of contraction in the hearts of vertebrates; and that large excess of $CaCl_2$ causes arrest in diastole in the former and arrest in systole in the latter. The question now arises as to how we may account for these differences.

Seliskar (1914) showed that the excitability of the ventricle of the turtle increases with decrease in concentration of $CaCl_2$. Ringer and others have found that excess of $CaCl_2$ causes decrease in frequency in the heart of the frog; and Ringer (1882), Locke and Rosenheim (1907), Mines (1912), and others have shown that calcium is necessary for contraction of the vertebrate heart and that excess of Ca causes increase in the force of contraction, resulting, in high concentration, in arrest in systole. From these results Clark (1927) has concluded that calcium acts specifically on each of two different systems in the heart, one associated with excitation, and the other with contraction; and that it depresses the former and augments the latter.

If calcium affects these two systems in the heart in different proportions in different animals, the fact that $CaCl_2$ in high concentrations causes arrest in diastole in the heart of the oyster and arrest in systole in the hearts of vertebrates is probably due to difference in the extent to which calcium affects excitation and contraction in the two forms; i.e., large excess probably produces in the heart of the oyster complete block of automatic excitation, resulting in immediate arrest in diastole, so that the effect of calcium on contraction remains latent until automatic excitation is resumed on return to van't Hoff solution, when excessive contraction obtains; and large excess probably produces in the hearts of vertebrates only slight depression of excitation, allowing the more marked effect on contraction to be manifested by arrest in systole.

Locke and Rosenheim (1907), Mines (1912), and others—by means of electric records taken during the diastolic arrest of the frog's heart induced by pure solutions of NaCl or KCl—have shown that the normal rhythm of excitation continues for some time in

these solutions, although the muscle fibers fail to respond; and they conclude that sodium and potassium have an inhibitory effect on contraction. Libbrecht (1920), Pond (1920), and others have shown that lack of NaCl or KCl stops automatic excitation without impairing the ability of the muscle to contract, and they maintain that these salts also have a stimulatory effect on excitation.

If these two salts affect excitation and contraction in different proportions in the hearts of different animals, the fact that excess of either salt causes in the oyster arrest in systole, and in vertebrates arrest in diastole, is probably referable to the difference in the degree of their influence on excitation and contraction in the two forms; i.e., excess probably produces in the heart of the oyster overstimulation of excitation without much inhibition of contraction, resulting in arrest in systole; while in the hearts of vertebrates the general effect is slight stimulation of excitation and great inhibition of contraction, resulting in diastolic arrest.

If both the depressing effect of calcium and the stimulating effect of sodium and potassium on excitation are exaggerated in the heart of the oyster, and similarly the effects of these elements on contraction are diminished, perfect antagonism between the effects of calcium and the effects of sodium plus potassium would obtain, these conditions being necessary for the proper functioning of the tissue. The foregoing results indicate that these conditions actually exist.

SUMMARY

1. The effects of NaCl, KCl, $CaCl_2$, and $MgCl_2$ were studied in the heart of the oyster by perfusing it with solutions of each salt in several concentrations, and with van't Hoff solution containing an excess or deficiency of each salt.

2. Solutions of NaCl and KCl inhibit relaxation and cause arrest in systole in high concentration. The extent of contraction at arrest varies directly with the concentration.

3. There is an optimum concentration of KCl in van't Hoff solution, and either increase or decrease in this concentration inhibits relaxation.

4. Solutions of $CaCl_2$ cause decrease in frequency of beat and increase in amplitude of contraction in low concentrations, and arrest in diastole in high concentrations. The decrease in frequency and the increase in amplitude of contraction vary directly with the concentration.

5. Excess of $CaCl_2$ in van't Hoff solution causes increase in amplitude and decrease in frequency, while deficiency of $CaCl_2$ in van't Hoff solution causes inhibition of relaxation and inhibition of contraction; and complete lack causes arrest in diastole.

6. Solutions of $MgCl_2$, like those of $CaCl_2$, produce arrest in diastole in high concentration, but they do not cause increase in amplitude of contraction as solutions of $CaCl_2$ do.

7. Complete lack of magnesium salts causes slight inhibition of relaxation.

8. An explanation is offered for the apparently opposite effects of solutions of NaCl, KCl, and $CaCl_2$ on the vertebrate heart and on the oyster heart.

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THE NORMAL SPONTANEITY OF MOVEMENT OF THE RESPIRATORY MUSCLES OF THYONE BRIAREUS LESEUR[1]

(Ten figures)

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HOLOTHURIAN anatomy, gross and microscopic, has long ago been investigated and the essential facts are known to everyone. Instances of such studies are those by Gerould (1896) on *Caudina arenata*, and by Haanen (1914) on *Mesothuria intestinalis*. So, also, certain aspects of holothurian physiology, including respiration, have been carefully observed and reported, e.g., those by Henri (1903), Pearse (1908), Winterstein (1909), Crozier (1916, 1920), van der Heyde (1922), and Lutz (1930). In so far as these pertain to the present study, they will be cited in the following account of recent observations.

Crozier (1916), in his experimental analysis of respiration in *Stichopus moebii*, very correctly says: "The pulsations of the cloaca exhibit a high degree of coordination in the action of a number of individual effectors." His investigation was painstakingly done in so far as muscular movements could be determined by direct observation, and his study of normal anal and cloacal movements was supplemented by noting the effect of a series of modifying chemical solutions and differing temperatures. More recently Lutz (1930) has sought the cause of the rhythmic activity of the cloaca of the same form with oxygen-deficiency effects especially in mind; he used the circular cloacal muscles of *S. moebii* and *Cucumaria frondosa* in his determinations.

The point to the present study is not critically to retravel ground already covered by others, as this seems adequately done from the standpoint of their objectives, but to analyze the quite involved respiratory mechanism by giving each factor (muscle group), so far as possible, a chance to demonstrate its own independent activity, and subsequently to note the behavior of them all in combination, simulating, it is hoped, something of their resultant action in an unmutilated animal. Quite in advance of such analysis, it is entirely conceivable that some sets of muscles may be neutral and inactive save as instigated by movements of adjacent tissues, or actual nerve impulses; and this has turned out to be the fact.

MATERIAL AND METHODS

The holothurian used was *Thyone briareus* Leseur, found abundantly on shallowly submerged mud flats at Woods Hole. Portions of the muscle system being investigated

[1] There is serious question, doubtless, whether the term "spontaneous" can be applied with strict accuracy to the contractile movements of any adult metazoan muscle bundle; for such is essentially always a combination of muscle and nerve tissue; if entire nerve-cell bodies are not involved, extensions from such are. It is true, nevertheless, that custom has permitted such phrases as "spontaneous muscle action," especially with reference to smooth muscle, e.g., of the intestine, or bladder. It is in this sense, indorsed by usage, that the term, as adjective or noun, is employed here.

were excised, suspended at one end by a glass hook, the other end being connected to a delicately balanced "heart lever" for kymographic recording. In some instances a lever was devised from a light grass straw, and this further counterbalanced to catch any slightest movement, inertia and friction being reduced to a minimum. This "dictaphone method" of letting muscles tell their own tales—these amplified by using a lever with arms of very unequal lengths—was assumed to show up most of the facts in a given case.

Throughout any experiment the preparation was kept submerged in a beaker of sea water at a temperature practically the same as that in which the animals were living. It is not true, as formerly thought, that the coelomic fluid of echinoderms is completely identical with sea water, for the latter has a slightly higher pH; but as the objective of these observations was not the exact form of any particular contraction curve, but

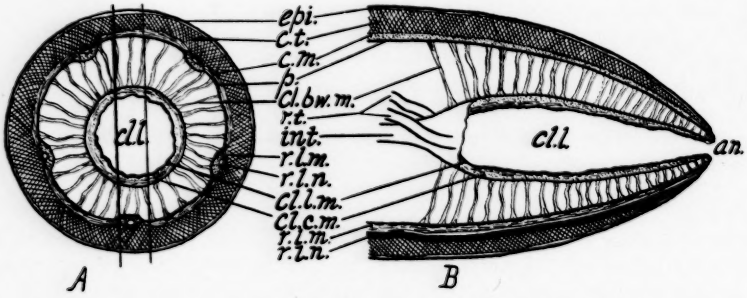

FIG. 1.—Diagrammatic cross-sections and longitudinal sections through *Thyone briareus* in the cloacal region to show musculatures concerned with respiratory intake and outgo. (*A*) cross-section; (*B*) longitudinal section: *an.*, anus; *c.m.*, circular muscle; *c.t.*, connective tissue; *cl.l.*, cloacal lumen; *cl.c.m.*, cloacal longitudinal muscle; *cl.c.m.*, cloacal circular muscle; *cl.bw.m.*, cloaca body-wall muscle; *epi.*, epidermis; *int.*, intestine; *p.*, peritoneum; *r.l.m.*, "radial" longitudinal muscle; *r.l.n.*, "radial" longitudinal nerve; *r.t.* "respiratory trees" (origins of).

rather its general occurrence and character, sea water was accepted as well adapted; Lutz (1930) did the same. Preparations lived vigorously over 50 hours under these circumstances; mutilated holothurians regenerate rapidly in sea water. To check on the pH question, in several instances CaCO in appropriate amount was added in the middle of the experiment, thus making any modification of behavior contrastingly apparent; but rate and amplitude seemed affected in indeterminate manner if at all.

As a convenient reminder of the arrangement of muscles concerned with respiration in a holothurian, line figures of the portion of anatomy involved are here inserted (see Fig. 1). Following the nomenclature adopted in the legend, it is obvious that the following classification can be made:

A. Mechanisms which, individually or in combination, may effect expulsion of water from the cloaca
   1. Circular muscles in the cloaca
   2. Longitudinal muscles in the cloaca
   3. Circular muscles in the body wall
   4. Longitudinal muscles in the body wall

5. Muscles in the "respiratory trees" and their end bulbs.
Number 3 and 4 can be effective because the coelom is full of fluid and compression of this will be transferred to all intracoelomic structures.

B. Mechanisms involved in the expansion of the cloaca and thus in the respiratory intake of that organ
1. General relaxation of the body-wall tissues, including muscles there present, with consequent release of pressure on all organs in the coelom
2. Active contraction of the numerous separate muscle bundles leading from the cloacal to the body wall

RESPIRATORY RHYTHM

In line with what other observers on related material have said, it may be mentioned in advance of any special description that whichever of the above-mentioned muscles, or whatever combination of them is active, that action is rhythmical. Such is true, of course, in the functioning of many respiratory mechanisms, though not with respect to the external or larval gills of some amphibia, the epidermis of worms, the gills of mollusks, or the branchiae of many echinoderms. Hence, the fact of an expected rhythmicity may well be in mind while examining any respiratory phenomena.

As for holothurians, Pearse (1908) noted an average interval of 39 seconds between each two of 144 successive "spouts" of *Thyone*, though this rate was far slower in other cases. Crozier (1916) gives data to show that the rate of respiratory movement in *Stichopus* is directly correlated with, proportional to, the size of the individual; and he notes that there are numerous minor contractions (inspiratory?) of the cloaca between each two expelling movements.

CONTRACTILE BEHAVIOR OF MUSCLE SYSTEMS

A. MUSCLES INDIVIDUALLY OR COLLECTIVELY FAVORING EXPULSION OF WATER

1. *Circular cloacal muscles.*—If a ring of cloacal wall is made by transverse section of any portion of that organ, not including any part of the anal area, and such preparation is so suspended as to record its movements on a kymographic surface, such a muscle ring from *Thyone* exhibits no contractions even though left for hours. This is so even though the setup is such that essentially no strain is present; true also if the ring is severed and mounted as a flat strip. However, a prolonged relaxation, lengthening, is characteristic of such a preparation. Figure 2 is a reproduction of the first 8 hours of a 28-hour graph, and is wholly typical. The obvious conclusion is that the circular cloacal musculature does not, of itself, initiate or effect the expiratory outflow.

This finding does not agree with Lutz's (1930) account of his similar study of *Stichopus;* in that form such a preparation apparently shows activity at once on setup. It does agree with Crozier's (1916) conclusion that "the stimulus to spouting has its origin outside the cloaca"; and as favoring this latter opinion, if a recording circular cloacal strip has adhering to it, by normal support through the radiating cloaca-body wall-muscle strands, a portion of the body wall (perhaps only peritoneum), including some fragment of a longitudinal nerve or its derivatives, then, after a prolonged relaxation phase, contractions set in and continue for many hours. Figure 3*a* illustrates such a case, the hours being 1–3 inclusive, and 7–9 inclusive, after attachment to the recording lever. Such mounts may remain active for upward of 50 hours.

If a further preparation, intended to betray the effect of anal-ring tissue on the

activity of adjacent circular cloacal muscle, is set up and a faster drum used, the result may be as in Figure 3*b*. This in turn should be compared with Figure 3*c*, which is a slow-drum graph obtained by using the entire anal sphincter ring, no cloacal wall included. The special point of interest in *b* is not any time-rate difference between the major contractions in it compared with those in *a*, for in that respect they essentially agree; but they are unlike in the presence of several lesser movements between each two major ones in *b*. These are interpreted as due to cloacal expulsion contractions, several of which occur to one anal closure. This is further substantiated by the curve *c* in this figure, which records the behavior of anal sphincter muscles only.

There is thus an interesting gradation of action of the circular cloacal musculature: by itself it develops no "spontaneous" movements; if connected with a longitudinal nerve bundle, much action is the rule; a similar ring in connection with anal tissue develops a double set of contraction crests, an interpretation of which has been ventured; and, finally, the anal ring has its own opening and closing program.

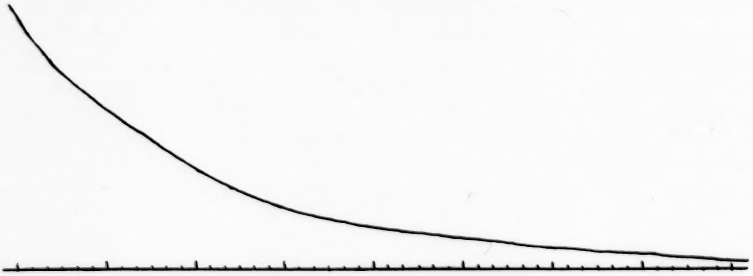

FIG. 2.—Relaxation curve of circular cloacal muscle of *Thyone;* no "spontaneous contractions." Time curve, 10-minute intervals, grouped into hours. Total time, 8 hours.

2. *Cloacal longitudinal muscles.*—In this series of setups the cloaca was used either as a whole, or, in other instances split open and mounted as a flat preparation. This latter procedure is probably the more reliable since by such method the complicating influence of circular muscles at right angles is perhaps lessened. As in the instance of experiments with circular muscles, only prolonged relaxation occurs; no contractions. Figure 4*a* is a reproduction of the first 8 hours of a 25-hour graph. That the conditions during such a period were favorable for activity is evidenced by the fact that the preparation responds at once, after long inactivity, to any ordinarily used stimulus, even mechanical disturbances.

The further story of longitudinal cloacal muscle when it is accompanied by its normal attachments to the body wall, and thus to the nerve bundles, is essentially like that told of circular muscles. Figure 4*b* is a graph written by such a preparation; the upper of the two curves began 3 hours and 20 minutes after setup, the lower 4 hours and 6 minutes after setup. The behavior of longitudinal cloacal muscle is further similar to circular cloacal muscle when a portion or the whole of the anal ring is in connection with it. Figure 5 is a reproduction of the action story of such a mount. Thus it is plain that stimuli may spread into the cloacal wall from the anal ring and activate either circular or longitudinal muscle elements.

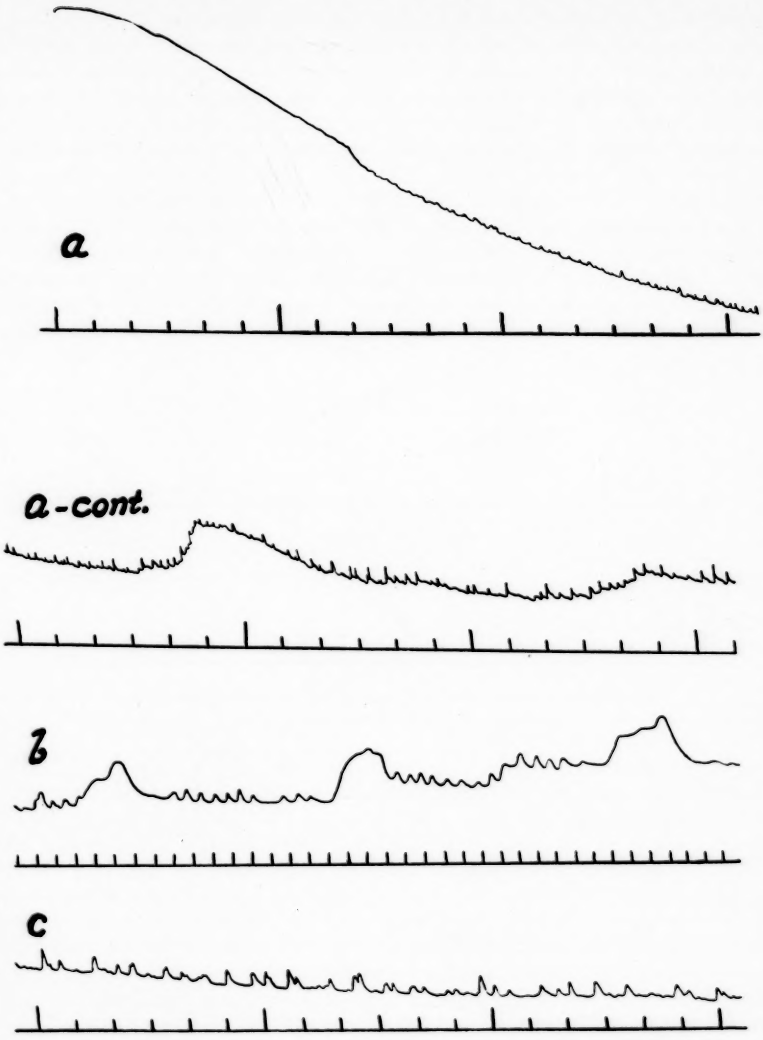

FIG. 3.—(*a* and *a cont.*) Contractile behavior of circular cloacal muscle when connected with main longitudinal nerve. Time curve, 10-minute intervals, grouped into hours. (*b*) Contraction curve of posterior circular cloacal muscle plus portion of anal ring. Time curve, 15-second intervals. (*c*) Contraction curve of anal-ring muscle. Time curve, 10-minute intervals, grouped into hours.

In *Stichopus*, according to Crozier (1916), the rate of pulsation of the anterior cloacal muscles is faster than that of the muscles nearer the posterior end. In a number of cases sufficient to justify the statement, the present study has evidenced a similar probability. In a particular instance the average rate per hour for 3 hours of an anterior piece was 43; of the posterior piece, 25. Again, however, much allowance must be made for individual differences of behavior, and the special rate mentioned would vary in either direction.

3. *Circular muscles of the body wall.*—In testing these, the peritoneum and all structures internal to it, also longitudinal muscle bundles and nerves, were dissected away. Only the parietal wall, on which the circular muscles are firmly imbedded, was used. No preparations of this sort showed any "spontaneous" contractions whatever; only slow and limited relaxation, lengthening, although observed for as much as 10 hours.

The outcome is quite otherwise, however, if the peritoneum and portions of any of the five longitudinal nerves are left attached to the circular piece excised. The sample

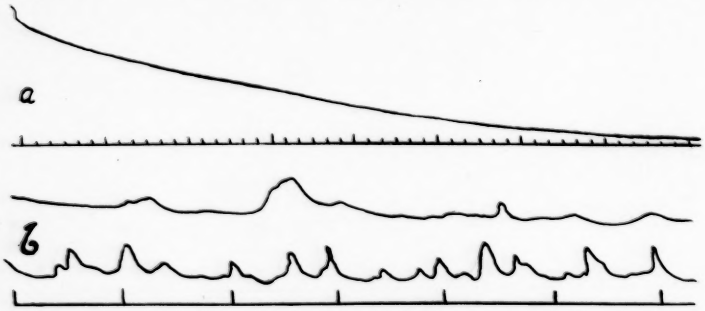

FIG. 4.—(*a*) Eight-hour relaxation curve of longitudinal cloacal muscle. Time curve, 10 minutes, grouped into hours. (*b*) Contractions of longitudinal cloacal muscle when connected to body-wall nerves. Time curve, 1-minute intervals.

here reported was taken about 1 cm. anterior to the anus. Like practically every other contractile tissue employed in these observations, a considerable relaxation follows the setting-up process; in this instance (Fig. 6) this lasted about 2 hours and 20 minutes, after which weak contractions began. The amplitude increases and a true rhythm is followed by irregular behavior until such a record as here shown results, after times specified in the legend. Such a performance, of course, varies much as to detail in repetitions of the setup, but the instance here used is truly characteristic; as implied above, it must doubtless be evaluated in terms of the longitudinal nerve trunk, not in those of its own spontaneity. This belief is further substantiated as one subjects a longitudinal muscle bundle to a similar test.

4. *Longitudinal muscles of the body wall.*—As is well known, longitudinal parietal muscles, aside from those generally referred to as "radial longitudinal muscle bundles," are not supposed to exist. However, the connective tissue layer of the body wall is so heavy that one wonders if perhaps some muscle fibers may not be scattered among them; hence, preparations have been made for use in carefully checking up on any movements which might possibly show themselves in strips taken in a longitudinal plane, freed from peritoneum, radial longitudinal muscles, and radial longitudinal nerves. Such prepara-

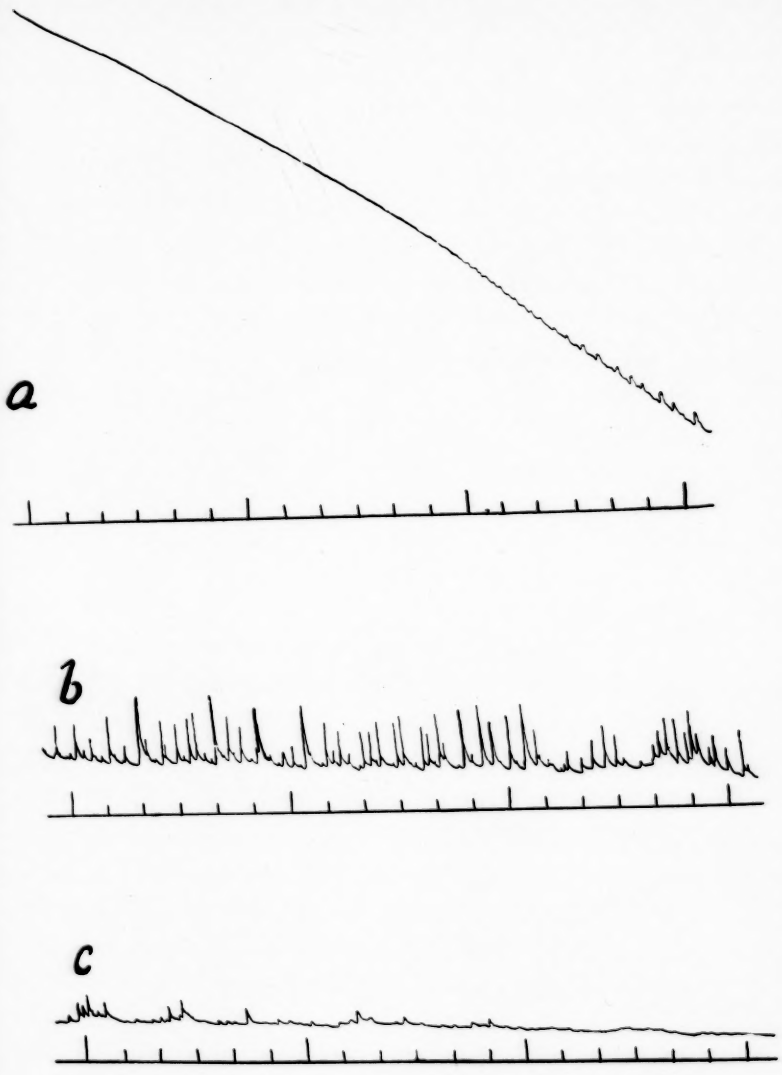

FIG. 5.—Contraction curve of longitudinal cloacal muscle when connected with the anal ring. (*a*) Hours 1–3 inclusive. (*b*) Hours 10–12 inclusive. (*c*) Hours 15–17 inclusive. Time curve, 10 minutes, grouped into hours.

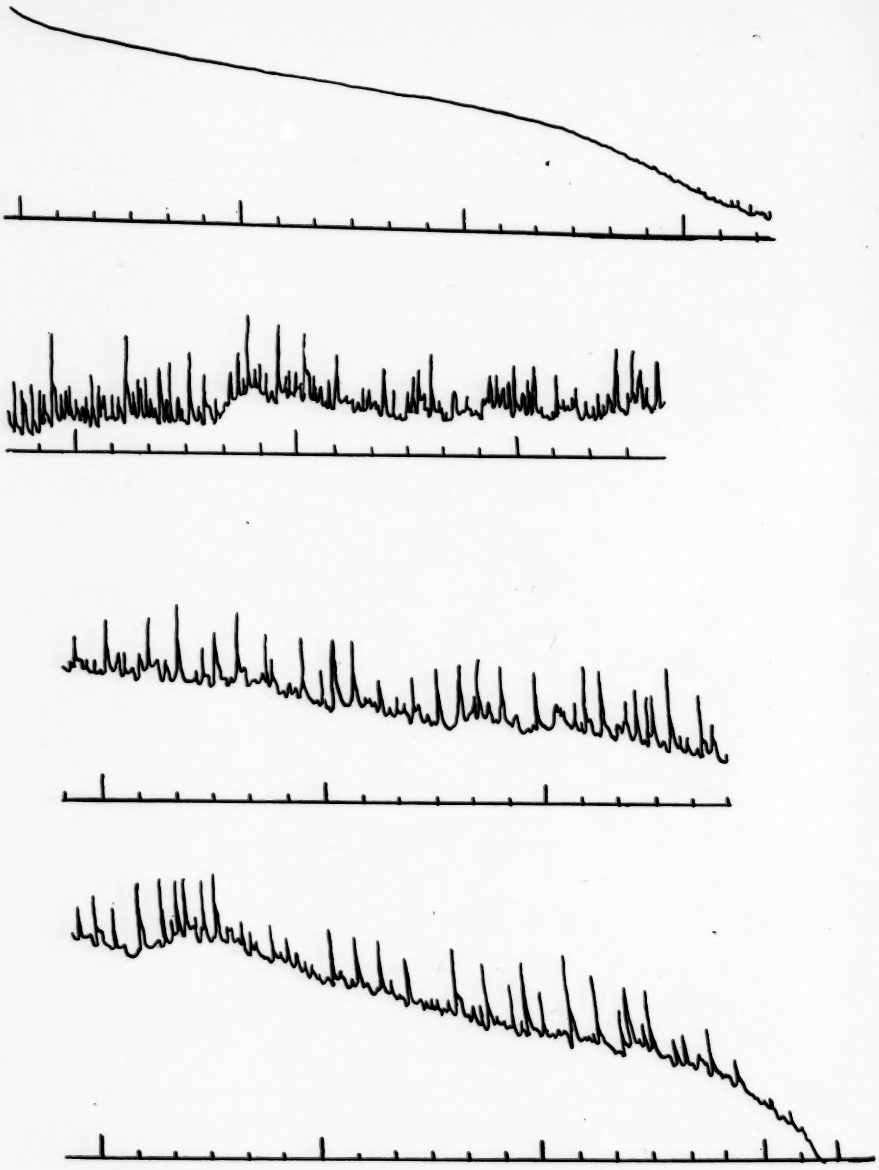

FIG. 6.—Contractile behavior of circular body-wall muscle of *Thyone;* when associated normally with peritoneum and nerve tissues. Portions of one record; hours 1–3, 6–9, 14–17, 17–20½. Time curve, 10 minutes, grouped into hours.

tions respond only with great elongation, even when a minimum load is used; no actual muscle-like shortening appears.

The "radial longitudinal muscle bundles" are so compact and workably large, even in so small an animal as *Thyone*, that they are easily separated from other tissues, either in conjunction with the accompanying nerves, or the muscle bundle may be split and the nerve be left behind with a muscle remainder. As to the behavior of a muscle strip alone, it manifests no contracting tendencies; a typical graph shows relaxation for about $4\frac{1}{4}$ hours, followed by slight improvement in tone for about 5 hours, after which loss of tone occurred until breaking. In contrast, if nerve and muscle both are mounted, a vigorous and prolonged series of contractions sets in. Figure 7 is a reproduction of a portion of a graph written by such a preparation.

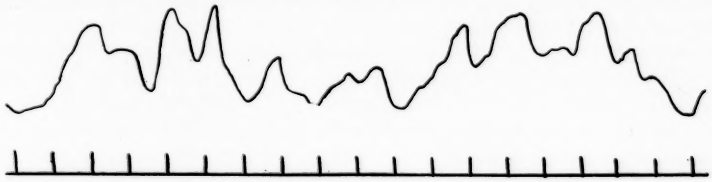

FIG. 7.—Contraction record of a longitudinal muscle bundle from body wall of *Thyone;* in normal association with nerve. Time curve, 30-second intervals.

5. *Contractions of "respiratory trees."*—It is wholly easy to note that isolated portions of the "respiratory trees" show movements over long periods; in one test a terminal twig was active on the third day after excision. The stems of the "trees" are contractile, as are also the walls of the end bulbs. To a degree each bulb carries on its own irregular system of expansion and shrinking. Numerous attempts were made to reduce these movements to graphic form, and to learn thereby if any periodicity or other specially characteristic movement pattern obtains in these structures; but all mechanical devices tried by the writer proved inadequate to the delicacy of the situation. Figure 8 is sub-

FIG. 8.—Contractile movements of a twig of the "respiratory-tree." Time curve, 15-second intervals

mitted, though quite uninforming beyond its evidence of independent change of form. This was secured by placing a bit of paper $\frac{1}{4}$-in. square on a group of end bulbs and carrying the motion to a recording lever by a delicate split straw, the weight of which was pendent from the lever. It should be easily possible to secure excellent moving pictures of respiratory-tree behavior.

Further, it is clear that the large stems of the "trees" change their length little save under special stimuli; they have been most carefully mounted, essentially all tension avoided, but have furnished no satisfactory action graphs. Any mechanical disturbance—e.g., a slight water current, suddenly applied—will elicit a response; this is soon followed by slow relaxation and elongation. The end bulbs, in contrast, appear quite independent of more central stimulating centers for initiation of movement.

B. MECHANISMS INVOLVED IN EXPANSION OF THE CLOACA, AND THUS IN THE RESPIRATORY INTAKE OF THAT ORGAN

1. *General relaxation of body-wall tissues.*—In the classification of respiratory mechanisms listed in the earlier portion of this paper, enlargement of the cloaca and thus conditions inducing flow of water into its cavity have been indicated as favored by a general relaxation of all muscles and tissues except the set radiating from cloaca to body wall. This statement is hardly susceptible of proof on account of the numerous factors involved; but the validity of such assumption can hardly be doubted since the situation is covered by applying the simplest principles of hydrodynamics.

2. *Active contraction of fibers leading radially from the cloaca to the body wall.*—Arranged as these muscle fibers are (Fig. 1), i.e., attached at their inner ends by insertions scattered in large numbers over the entire outer surface of the cloaca, while their outer

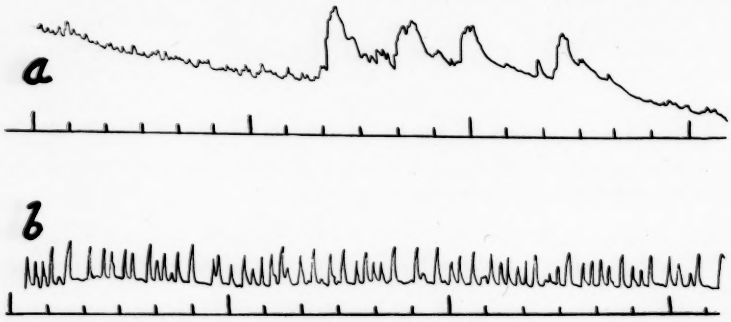

FIG. 9.—Kymographic records of muscle fibers leading from the cloaca to the body wall: (*a*) of fibers between cloaca and area between main longitudinal nerves, (*b*) of fibers between cloaca and area with longitudinal nerve. Time curve, 10-minute intervals, grouped into hours.

ends are anchored in the body wall, one cannot doubt that their contractions enlarge the cloacal lumen and thus, when the anus is open, induce an inflow of water. If, then, a preparation is made for kymographic purposes by excising a small area of the body wall and similarly an area of the cloaca, in the same radius, to which muscle fibers from the former are attached, and these two areas be used as terminals for suspension purposes, records of action on the part of these readiating muscle fibers are easily secured.

In Figure 9 two curves are shown: (*a*) action when the body-wall terminus is taken from an interradial area, thus not involving direct association with a main radial nerve, and (*b*) a result when the distal end was an area including a portion of a radial nerve. Both setups furnish contractions aplenty, although they occur rather leisurely, varying in rate from one in 4 minutes to one in 10 minutes. In all instances contraction is more rapid than is relaxation, thus typically of the muscle manner. On the whole, fibers inserted along the nerve-trunk area are given to more rapid and more orderly activity than are those associated with interradial surfaces; they also enter on their rhythmic role more promptly after excision. The apparently disconcerted character of contractions of interradial fibers is not herewith given interpretation; it might be attributed to "surgical shock" were it not that the sample of behavior here submitted is that of $17\frac{1}{2}$–20 hours after setup.

A AND B. ACTION OF THE COMBINED INSPIRATION-EXPIRATION MUSCLE COMPLEX

If a preparation including the anatomical features occurring between the parallel lines in Figure 1 (A) is made, suspension hooks attached to the oppositely located portions of the body wall, and proper connections to a recording lever be made, graphs on the order of that shown in Figure 10 may be secured. The usual inert phase occurs, this being followed by a very persistent series of contractions, in this case lasting upward of 56 hours. A like result is consequent if the integument and connective tissue of the body wall are eliminated. Analysis of what is revealed by such a movement complex will contribute to its interpretation.

Reference to Figure 10 permits the following summary: After a relaxation period of about 4 hours, feeble contractions set in; in about 8 more hours these attained practically one-quarter the maximum later reached; a half-maximum was attained in another 5 hours, the maximum activity being reached after a further 7 hours, i.e., about 24 hours after excising and setup was completed. This maximum performance was maintained about 4 hours, and followed by a very gradually lessening action during a further 26 hours; the entire active period in this instance thus totaled approximately 56 hours. Perhaps this may be taken as a fairly concrete example of the great tenacity for life which holothurians, as wholes or in parts, under adverse circumstances, e.g., autoevisceration or great artificial mutilation, seem able to demonstrate.

A striking feature of the record in Figure 10 is the grouping of contractions which obtains throughout the entire period of action. About 4 hours after contraction sets in, the number in a group was four to nine; 10 hours after beginning, ten to eighteen; an average of twenty-three to twenty-four contractions occurs in groups at 18 hours; at the period of maximum action these are sometimes in groups of as many as forty-four components. The larger number tends to be retained as the long record goes on, but amplitude diminishes, and finally the contractions become minimum and irregular. These groups constitute an obvious and unusually striking periodicity. It is equally interesting, furthermore, that each of the major aggregates is, in turn, divided into subrhythm groups so constantly as to establish a further characteristic of this complicated movement phenomenon. These subgroups consist of a differing number of curves, perhaps only two—a smaller and a larger in earlier periods—but of three to six in the period of maximum amplitude; and they continue to be a feature of the behavior of this muscle complex practically to the very end of its contracting.

What is the interpretation of such an action record? The mechanism in this setup is essentially a combination of all the muscle systems described individually in the earlier part of this study, inspiratory and expiratory; and in some manner each previously shown curve is involved in this, though one may not count on identification of all such separate components; the "mechanism-as-a-whole" is not simply the sum of its parts, especially not in its functioning. However, a helpful clue to the answer to our question can be most reasonably sought in a review of what actually happens in a normal living animal as the water currents enter and leave the cloaca. As previously noted, Pearse (1908) kept track of the time interval between "spoutings," and noted an average of 39 seconds between each two of 144 consecutive "spouts," though this rate was far less in some other specimens. Crozier (1916) gives data to show that the rate of respiratory movement in *Stichopus* is directly correlated with, proportional to, the size of the individual; and he notes that there are numerous minor contractions (inspiratory) of the cloaca between each two expelling movements. Lutz's (1930) kymograph tracings agree

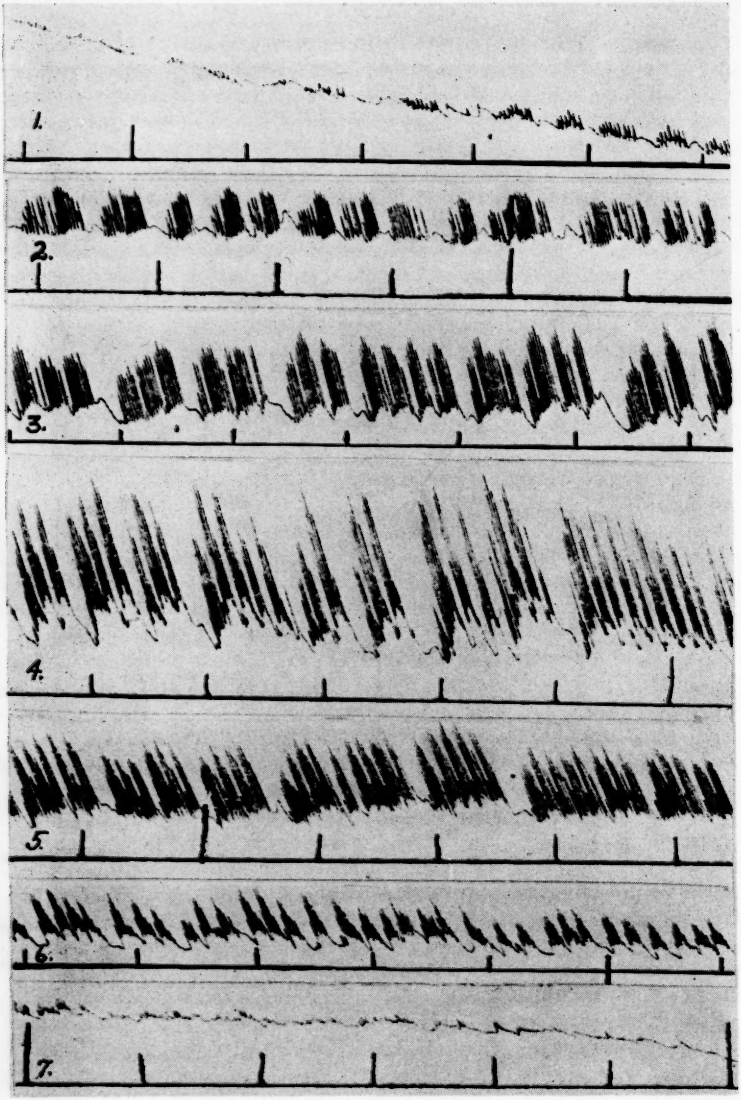

FIG. 10.—Photographic enlargement of portions of a 56-hour kymographic record of contractions of muscles included between parallel lines in Fig. 1 (*A*), mounted as single preparation. Approximate hour position in entire tracing of foregoing excerpts: *1*, 4–5; *2*, 11–12; *3*, 19–20; *4*, 26–27; *5*, 39–40; *6*, 49–50; *7*, 54–55. Time curve, 10-minute intervals.

with Crozier's figures that the rate in *Stichopus* is about one expulsion each 5–7 seconds. It is a mere chance, of course, that the slow rate in small *Thyone* and the faster rate in large *Stichopus* is in accord with Crozier's correlation noted above; for the assignment of these two animals to different genera was based on radical differences in anatomy and the accompanying physiological problems.

For the sake of illustrating the inconstant rate of respiratory "breaths" in *T. briareus*, the following table of stop-watch observations is given. The animal was roughly 8 cm. long, an average size, and was lying on sand in a dish of fresh sea water, the cloacal end barely submerged, the anterior end unirritated, tentacles extended; carmine granules

TABLE 1

TIME INTERVALS BETWEEN INITIATION OF EXPULSION STREAMS IN THE RESPIRATORY RHYTHM OF *Thyone briareus;* MEASURED IN SECONDS DURING A 1-HOUR PERIOD; TEMPERATURE OF WATER, 22.2 C.

10	18	16	18	24	58	25
50	23	18	25	18	45	16
23	24	20	17	16	103	11
17	18	32	60	17	55	28
105	17	32	175	18	27	16
27	16	21	24	18	25	10
15	16	9	10	7	24	17
26	16	9	18	12	25	22
67	53	8	17	17	28	18
31	11*	76	60	102	55	16‡
49	10*	33	25	14	47†	17‡
12	10*	91	25	24	19†	16‡
16	82	72	17	26	17†	10
15	48	78	18	12	30	17
14	27	14	18	15	32	90
17	27	7	95	17	85	84
16	26	58	31			

*During these periods there was present a very sharp annular constriction of the outer body wall at about the level of the anterior end of the cloaca.

†During these intervals the tentacles were being withdrawn; during the very next period (30) the tentacles were quickly extended.

‡During these intervals the tentacles were completely withdrawn. In all other instances the tentacles were extended save for the single one being turned into the mouth as per habit.

were in suspension about the anal region, and a lens was used to detect any change in water-level, or currents at the cloacal opening; the record is felt to be accurate for the given individual and occasion.

If now this table of respiratory frequencies is compared with the graph in Figure 10, it becomes evident that each contraction there recorded is an expulsion movement. The two lines of data cannot be completely reconciled, of course, derived as they were from different specimens, at different times of the year, with different temperatures of the surrounding water influential; but the range of respiratory rates differs so much in different individuals, as noted by both Pearse (1908) and Crozier (1916) as well as by the writer, that correlation is still easily made.

The first inclination of the writer was to presume the subrhythms to be instances of what Crozier observed in *Stichopus*, which shows 5–10 "cloacal pulsations" between

"spoutings." Such cannot be the fact in this instance, however, since the rate of spouting in *Thyone* as shown in Table 1 is readily applied to the graphic record here under analysis. So much care was taken in making this setup, essentially all tension avoided, that "pulsations" could hardly escape recording their presence. The difficulty in the case lies in the fact of so many possibilities; but the writer ventures to make this suggestion, the rate of subrhythms is not specially unlike the contraction rate of the radial cloaca-body wall muscle fibers as illustrated in Figure 9. Furthermore such explanation seems fairly justified by the fact that each set of contraction crests in each subrhythmical group shows a base-line level which has the form of a contraction curve. The periods between the major periodicity groups are believed due to a pause during which the respiratory trees empty themselves posteriorly into the cloaca, all muscles opposed to such procedure being relaxed during these pauses and hence a drop in the general basal curve-level.

Whether the foregoing attempt to account for the character of its inscription is correct or not, certainly such a rhythm and periodicity of movement are equal to any requirements a physiologically vital function may entail.

In his studies of *Stichopus*, Lutz (1930) concludes that periodicity in the contraction rhythms is generally, perhaps always, due to oxygen deficiency or other interference with respiratory equilibrium; and his results seem quite in line with that inference. The evidence of *Thyone* on this point seems not to permit a like conclusion; this last graph is a case in point: the contractions appear in groups from the very start. The total weight of tissue did not exceed 0.25 gm.; this being the only respiring thing in 150 cc. of fresh sea water, oxygen deficiency would hardly be in force so early, or, if so, life would have departed much more promptly than it did, and exhaustion would have appeared much before that.

One question which Crozier (1916) attempted to answer in his *Stichopus* studies was: "At which end, anterior or posterior, do stimuli enter the cloaca and cause its more or less peristaltic rhythm?" This was not a point specially in mind during the observations here reported; but as bearing on the matter it may be mentioned that transverse portions of the cloaca of *Thyone*, taken at any level between the two ends, show no contractions when isolated. However, preparations from any level, plus normal connections with the radial nerves or their branches, show vigorous and prolonged activity. The inference from this is that stimuli may not enter the cloacal wall at any single level, but at any level. The order in which they enter, whether antero-posteriorly or postero-anteriorly, would be expected to conform to the occasion, i.e., whether water is being pushed into the "respiratory trees" or out of the anus.

SUMMARY

1. Circular and longitudinal muscles in the cloacal wall, circular and longitudinal muscles in the body wall, muscle strands leading from the cloaca to the body wall, and anal sphincter muscles constitute a complex mechanical equipment serving respiratory movements in holothurians, e.g., *T. briareus*.

2. The contraction habits of each set of muscles have been investigated by the kymograph method of self-registration.

3. Cloacal muscles, circular and longitudinal, when excised by themselves seem lacking in spontaneous activity, except for relaxation.

4. Body-wall muscles, circular and longitudinal, when carefully separated from the

main radial longitudinal nerve cords and their derivatives, seem lacking spontaneous activity, except for relaxation.

5. Both cloacal and body-wall muscles, when accompanied by portions of the main nerve cords or derivatives of them, show long-continued spontaneous contractions; such may continue more than 50 hours under laboratory conditions.

6. In *Thyone* each sort of contractile tissue seems characterized by a very long period of relaxation (lengthening) before entering on its series of rhythmic contractions; this inaction period may last upward of 6 hours.

7. The muscle fibers radiating from the cloaca to the body wall must be regarded as the principal agents in inducing inspiratory currents. These are given to much spontaneous contractility when used with their insertions in the cloacal and body walls.

8. When an experimental setup involves portions of each respiratory musculature, a striking compound periodicity is superposed on a very prolonged rhythmical contraction habit. An attempt is made to analyze and account for this periodicity.

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THE EFFECTS OF EXPOSURE TO LOW TEMPERATURE ON THE DEVELOPMENTAL TIME OF EMBRYOS OF THE GRASSHOPPER MELANOPLUS DIFFERENTIALIS (ORTHOPTERA)

(Six figures)

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SINCE Bodine's (1932*a*) report of a developmental block ("diapause," according to the definition of Henneguy) in the embryos of *Melanoplus differentialis*, much attention has been directed to the morphology and physiology associated with this condition. Previous work of Carothers (1923), Bodine (1925, 1929), and Slifer (1930, 1931) led to the discovery of the diapause condition in this form, and work of Bodine (1932*b*, 1934), Bodine and Boell (1934*a*, 1934*b*, 1935, 1936), Boell (1935), Burkholder (1934), and Slifer (1932*a*, 1932*b*) have all dealt with some phase of the phenomenon.

Evidence has accumulated indicating that exposure to low temperatures breaks diapause, i.e., shortens the length of time at a constant high temperature required for the hatching of diapause eggs (Bodine, 1925, 1929, 1932*a*, 1932*b;* Slifer, 1932*b*). This effect of low temperatures has been incorporated by Bodine (1932*b*) into a theory regarding diapause as follows:

> Diapause eggs contain certain factors (*x*) which cause diapause in rather definite fashion and at definite stages in the egg's development. This is an "all or none type" of reaction. Diapause factors (*x*) are extremely susceptible to low temperatures and can be completely destroyed or inhibited by appropriate exposures to these temperatures. A slow gradual destruction or loss of potency of the diapause factors (*x*) occurs at constant high temperatures.

In the present work an attempt has been made to analyze what constitutes an "appropriate exposure to low temperatures" for the destruction or inhibition of diapause factors.[1]

MATERIAL AND METHODS

According to Slifer (1932*b*), who has made an intensive study of the embryology of *M. differentialis:*

> These [eggs], when kept at 25° C., develop for about three weeks. At this point an embryonic diapause sets in which may last for months. Growth, for unknown reasons, finally recommences, revolution of the embryo occurs, and the completely formed animal hatches about two weeks later. If, however, such eggs shortly after being laid are exposed to low temperatures, the diapause is prevented and, on return to a suitable high temperature, development proceeds without interruption to the end. . . . . For stages following revolution, or blastokinesis, eggs were used which had been allowed to remain buried in sand in outdoor cages from the time they were laid in September until January. These eggs when brought indoors had reached a stage of de-

[1] The author wishes to take this opportunity to express his appreciation to Professor J. H. Bodine for aid in selecting the problem and for suggestions during the course of the investigation, and to Dr. Eleanor H. Slifer, Dr. Gordon Marsh, and Dr. R. L. King for the various favors they have shown.

velopment corresponding to that of those kept twenty days at 25° C. from the time of laying. Hatching, in this case, occurred seventeen days later. The entire period of incubation may thus be said to have occupied the equivalent of thirty-seven days at 25° C. This is all the more interesting in view of the fact that occasionally *M. Diff.* eggs fail to "hibernate" and, when kept at 25° C., hatch about the thirty-eighth day.

Bodine (1925) and Parker (1930) have found that the period of incubation of grasshopper eggs previous to exposure to low temperature modifies the effectiveness of low temperature in stimulating development. In the present work the amount of incubation and the low temperatures to which the eggs were exposed have been varied to elucidate these points.

The experimental methods have been very simple. Eggs of the grasshopper, *M. differentialis*, have been used in the experiments. They were collected within 24 hours of laying, from females taken out of doors during the autumns of 1934 and 1935, and from the first filial generation of the 1934 females raised in the laboratory. Eggs at the desired stage of incubation were divided into groups. Some were kept at 28° C. as control material; others were exposed to low temperatures for given periods of time, then returned to 28° C., and the time of hatching was recorded.

The choice of morphological stages (degree of incubation), temperatures, and periods of exposure to low temperature has been largely arbitrary. Representative pre-diapause and early diapause stages have been used. They have been exposed for various intervals from 7 to 84 days to the low temperatures of 0°, 5°, 10°, and 15° C. maintained in (G.E.) electric refrigerators. When not at low temperatures they have been kept at the constant high temperature of 28°±0.5° C. in a covered container in a water bath regulated by electric heater, thermostat, and relay.

Several methods of caring for the eggs were tried and discarded. The two methods finally adopted were as follows:

1. Caring for small groups of eggs from a single pod. The eggs were removed from a pod into water, counted into groups of 25 each, and transferred onto moist cotton in the bottom of 15-cc. vials which were then stoppered loosely with cotton. One group from each pod was placed at 28° C.; others were distributed to experimental conditions. All vials were examined each day. Vials in which the surface of the eggs had become dry were moistened again. After nymphs began to appear in any vial, the empty shells and first exuviae were removed. Appropriate records were kept of eggs from an individual pod so that control and experimental groups could be compared.

2. Caring for large groups of eggs from several pods. In the experiments involving large samples the eggs from 50 to 107 pods laid on 1, 2, or 3 successive days were removed from the pods while submerged in water. The debris was removed by sifting the eggs through a sieve. By the time this had been accomplished, eggs from the various pods had been thoroughly mixed. Samples of 500 eggs were selected from the mixture by a volumetric procedure. After the sample was measured, the excess water was drained off and the eggs were mixed with 20–30 cc. of dry, sterile sand, which was then evenly moistened with approximately 2 cc. of water. This is sufficient moisture to make the sand damp, but not soaking wet. The eggs and sand were placed in a screw-topped bottle (75 cc.) and covered loosely with the top of the bottle. One sample from each mixture was placed at 28° C. as a control; the others were exposed to experimental conditions. The samples were examined regularly, and at intervals the eggs were sifted out of the sand and any spoiled ones were removed. They were then redistributed in clean

sand moistened as before. After hatching began the empty shells were removed when the eggs were sifted out of the sand.

Studies on the effects of moisture and relative humidity on the hatching time and viability of insect eggs (reviewed by Uvarov, 1931) have yielded a variety of results. The only generalization that can be made at present is that the results depend to a large extent upon the material used. Previous investigators working with grasshopper eggs have apparently experienced little difficulty in maintaining satisfactory conditions for this material (Bodine, 1925). Parker (1930) found that eggs of *Camnula pellucida* buried in moist soil, where the humidity must have been nearly 100 per cent and where contact moisture was available, showed a higher percentage hatching than eggs suspended in humid atmospheres. Excessive contact moisture (wet soil) was not so satisfactory, probably owing to lack of aëration, since Boell (1935) has shown that submergence in unaërated water retards development. Dry soil was unsatisfactory, as the eggs dried out and collapsed. In the present investigation the large samples were buried in moist soil. The small samples were not buried but were on the surface of moist cotton where contact moisture was available and the atmosphere was nearly saturated. Such samples have given as high as 100 per cent hatching.

RESULTS

During the course of the investigation hatching dates have been recorded for several thousand eggs. Only examples of typical cases and summaries of results are presented here.[2]

1. CONTROL GROUPS KEPT AT 28° C

Groups of eggs were originally set aside as controls from which to determine the average hatching time of the species as a basis for measuring the effectiveness of exposing eggs to low temperatures. These control groups have given results quite instructive in themselves. Marked differences have been found in eggs from different pods. Minimum hatching times of eggs from different pods kept only at 28° C. have varied from 32 to 107 days. Average hatching times for eggs from single pods show a similar spread. Values ranging from 50 to 135 days have been obtained.

If each record be taken as an independent random sample from an adequately large and homogeneous population, statistical statements of the characteristics of the population might be formulated. Such measures as the mean, standard deviation, and probable error of the mean are usually chosen for this purpose. Examination of the present data indicates that such values do not adequately characterize the population. Data are available on eggs from three seasons. The lines A', B', and C' of Figure 1 are sufficient to show that the values for the mean, standard deviation, etc., of each season would be distinct and characteristic. Further study of Figure 1 and of Figure 2, which summarizes data obtained from individual pods, shows that even within a given season the statistical measures are not adequate characteristics of the population. All pods collected early in a season vary in one direction from the mean while those collected late in a season vary in the opposite direction.

An unpublished communication from E. Cook of this laboratory shows that the first pod of eggs obtained from an isolated pair of grasshoppers of this species started hatch-

[2] The detailed data from which the selection has been made are on file in the Zoölogy Library of the State University of Iowa.

ing in 114 days, while the sixth pod started hatching in 44 days, actually 36 days before the first pod. More data from isolated individuals are being secured at present. In one case, at least, the last pod of eggs has started hatching before any of the other pods laid by the female. This suggests that the seasonal progression toward shorter hatching time, noted in the present investigation, was probably an expression of the same tendency. This cannot be stated definitely since many grasshoppers were kept in a single cage, and the only record kept was the date on which the eggs were collected.

Examination of hatching time among eggs from a single pod shows that different pods have distinct and characteristic types of distribution in this respect. Table 1, which gives the detailed data on distribution of hatching for two selected groups of 25 eggs from individual pods, indicates that in some pods the majority of the eggs hatch on 1 or 2 days, while in others hatching is distributed evenly or sporadically over a considerable period. Pods of eggs in which the majority hatch on 1 or 2 days are also characterized by having a small range between minimum and maximum hatching time, the mode and mean close together and near the minimum hatching time. Examination of Figure 2 shows that these conditions are found almost exclusively in pods laid in the last half of the 1934 season.

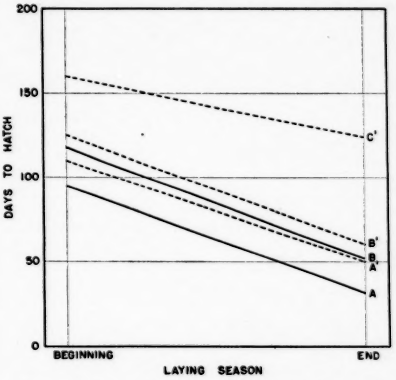

FIG. 1.—Smoothed curves showing a tendency, as the laying season progresses, toward shorter minimum hatching time (solid lines) and shorter average hatching time (dashed lines) of eggs kept continuously at 28° C. *A* and *A'*, control groups, spring of 1935; *B* and *B'*, control groups, autumn of 1934; *C'*, control groups, autumn of 1935.

Besides the variations in length of time required for hatching, variations in the percentage of eggs which do hatch have been recorded. While these seem to be more at random than the variations in hatching time, there is some correlation between percentage hatching and the length of time required for hatching. Thus, out of 68 records on individual pods which showed average hatching times below 100 days, there were 16 which have 80 per cent or more hatching; while out of 24 records on individual pods whose hatching times were over 100 days, there was only one group with 80 per cent hatching. Although this correlation between hatching time and percentage hatching is rough, it is in a direction which, in conjunction with data on eggs exposed to low temperatures, suggests that some eggs of this species never come out of diapause if kept at high temperatures. This condition was found by Carothers (1923) for *Circotettix*.

Owing to these variations in eggs kept only at 28° C., no attempt has been made to present values for the mean, standard deviation, or other statistical measures for the entire population. Each experiment performed has been considered in relation to the control group from the same source.

2. EFFECTS OF EXPOSURE TO LOW TEMPERATURES

The most obvious effect of exposing insects to low temperatures is to retard metabolic processes, even to complete inhibition. Numerous investigations of that temperature above which an insect can undergo development have led to the concept of the "develop-

mental zero," or "threshold of development." This concept has been developed very well by Sanderson and Peairs (1913). Bodine (1925) reports that developmental zero for the eggs of *M. differentialis* is 18° C. as measured both by individual experiments at low temperatures and by extrapolation after the method of Sanderson and Peairs.

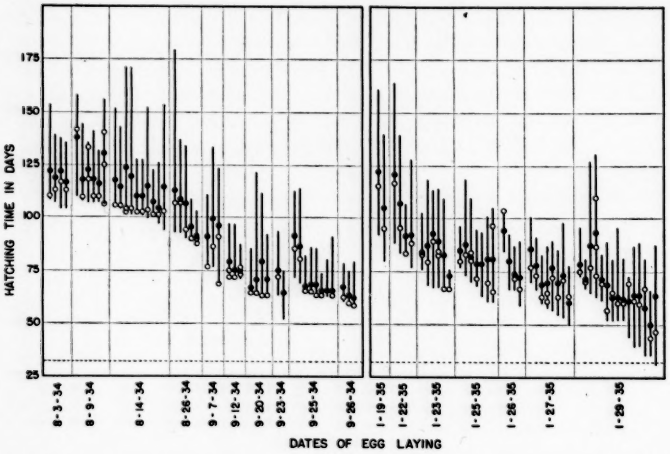

FIG. 2.—Summary of hatching data on control groups. Each vertical line represents data on 25 eggs from a single pod kept at 28° C. The lower and higher limits represent minimum and maximum hatching time; circles, modal hatching time; and dots, average hatching time. The groups are arranged in order of deposition from the first of each laying season to the end of the season.

TABLE 1

DISTRIBUTION OF HATCHING FOR SELECTED GROUPS OF 25 EGGS FROM 2 PODS

Date	Exp.	Days at 28° C. and, in Parentheses, the Number Hatching	Average Hatching Time	Percentage Hatching
1-22-33.....	1	88(1), 95(1), 97(1), 101(1), 105(2), 113(1), 114(2), 117(3), 118(1), 122(2), 130(1), 132(1), 137(1), 142(2), 148(1), 152(2), 165(1)	122.7	96.0
9-25-34.....	3e	64(9), 66(6), 68(1), 71(2), 75(1)	66.2	76.0

The occurrence of a dispause at high temperatures in this material, which was not appreciated in 1925, raises the question of whether this period should be excluded in considering the effect of temperature on development. Bodine (1925) indicates that the total time from egg laying to hatching at different constant temperatures (which includes diapause) shows the expected Q_{10} of 2.47. However, in the present investigation greater variations in total time have been observed among eggs kept only at 28° C. than were

reported by Bodine as temperature effects. More recent work of Bodine (1932 ff.) and others has indicated that the length of diapause is normally variable at a constant high temperature, but that the time spent in active development is quite constant. The present data have given a more comprehensive statement of the normal variations in length of diapause at a constant high temperature and have furnished some information on the effect of temperature on the actual developmental processes.

Examination of eggs placed at 15° C. immediately after laying reveals that after 28–35 days the embryo can be removed from the chorion. This cannot be accomplished until the inner chitinous membrane is laid down by the embryo, which occurs on the fifth or sixth day when eggs are kept at 28° C. This indicates that the embryo has developed to a corresponding stage while at 15° C. Eggs placed at the lower temperatures of 0°, 5°, and 10° C. and kept for similar periods, or even as long as 84 days, have never yielded to attempts to remove the chorion.

TABLE 2

AMOUNT OF DEVELOPMENT OCCURRING DURING 63 DAYS' EXPOSURE TO LOW TEMPERATURES

Temp. at Which Eggs Were Kept (Degrees Centigrade)	Time Required at 28° C. To Reach Same Morphological Stage as Shown by Eggs at Low Temp. (Days)	Number of Cases; Selected at Random from 500 Eggs
0	0	10
10	0	10
15	11	1
15	12	7
15	13–14	3

Table 2 compares the time required at 28° C. to reach a morphological condition similar to that obtained after 63 days' exposure to various low temperatures. The eggs were placed at low temperatures immediately after deposition and were dissected for determination of morphological development at the end of 63 days. Definite and extensive advances in development are made at 15° C. which do not appear at the lower temperatures with early eggs.

Slifer (1932*b*) has shown that the time involved in active development at the constant temperature of 25° C. is about 37 days. At 28° C. this is reduced to about 32 days. Data presented in Table 3 show that, if the embryos are exposed to low temperature before reaching the diapause condition, the time required at 28° C. may be reduced to a certain extent, while embryos which have reached the diapause condition do not show such reduction.

Both Tables 2 and 3 indicate that first-day embryos undergo considerable development at 15° C., very little at 10° C., and none at either 5° or 0° C. At 15° C. there is a limit to the amount of development possible. This is equal to about 15 days at 28°C., which coincides with the time necessary for the embryo to reach diapause, or with the time required for non-diapause embryos to reach a stage just before blastokinesis.

Fifteen degrees is below the threshold for the process of blastokinesis. Fourteen-day-old embryos have already approached this and show no indication of further development at any of the low temperatures used.

Less obvious than the effects of low temperature in retarding development are the effects on the appearance and duration of diapause exhibited by these embryos. Bodine (1925) and Parker (1930) have shown that, after sufficient exposure to low temperatures, the eggs of some species of grasshoppers require only a minimum time at high temperature to complete their development. Both have indicated that there are periods in the development of the embryo when the effectiveness of low temperature is greatest. Parker has indicated that 0° C. is more effective than 8° C. with the material he used.

An extensive series of experiments have been performed in order to determine the effect of periods of exposure to low temperature on the appearance and duration of diapause. Since variations in the length of time required for hatching at 28° C. are due

TABLE 3*

SHOWING REDUCTION IN TIME (DAYS) AT 28° C. BELOW THE EXPECTED TIME OF 32 DAYS AFTER FIRST-DAY EGGS (UNDER COL. A) AND FOURTEENTH-DAY EGGS (UNDER COL. B) WERE EXPOSED TO THE INDICATED LOW TEMPERATURES

Duration of Exposure (Days)	15° C.		10° C.		5° C.	
	A	B	A	B	A	B
35	10(11)	0(1)	8(5)	0(4)	0(1)	0(4)
56	15(20)	.....	7(6)	.....	.....	0(3)
84	15(10)	0(100)	5(2)	0(213)	.....	0(1)

* In parentheses are the number of cases giving the response. Blanks indicate that all eggs exposed to the combination of temperature and exposure exhibited some diapause.

to differences in the length of diapause (except as previously noted), the time required at 28° C. is one satisfactory end point in the following experiments. It must be remembered, however, that a hatching time of about 32 days indicates no diapause present, and other values must be reduced proportionally to give the actual length of diapause under the conditions of the experiment. The data presented for eggs kept at 28° C. indicated large variations in the length of diapause; consequently, the effect of exposure to low temperature must be interpreted in relation to the characteristics of the eggs with which the experiment was performed. When the experiments were started, the variations in hatching time at high temperature were not fully realized. Series of experiments were planned using numerous pods of eggs, some of which had distinctly different characteristics. Although the data on control groups of eggs indicate an almost completely graded series showing average hatching time from 50 to 164 days, actually, when the experiments performed with these eggs are considered, it is possible to separate them into experiments performed on eggs with comparatively short hatching time (under 100 days) and with comparatively long hatching time (125–64 days). The results obtained when these different eggs were exposed to the same conditions were distinct. Data presented in Figure 3 will serve to indicate the relationships of the responses shown by the two types of eggs.

Lines A'' and B'' indicate the average hatching times of the control groups involved; lines A and B the hatching times (at 28° C.) of eggs of the two types exposed to 15° C. for the indicated period immediately after they were laid. While the figure shows that less time is required at 28° C. for both long and short diapause eggs after exposure to low temperatures, the absolute values are as characteristic as those of the control groups. Examination of the total time from the laying of the eggs until hatching, which includes the time of exposure to low temperature, shows a striking difference in the response of the two types of eggs. Lines A' and B' give this information. They show that with short diapause eggs the total time is never less than that of the control groups, while in the case of long diapause eggs the total time may be reduced to about half that of the control groups. This criterion of "total time" from egg laying to hatching is of particular value in analysis of the effect of exposure to low temperatures on the appearance and duration of diapause.

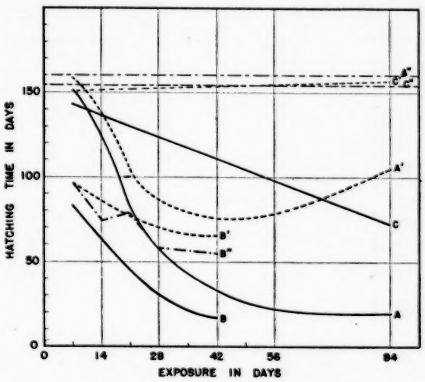

FIG. 3.—Showing effects of potential diapause on results obtained in similar experiments, and the relation between time required for hatching at 28° C. and the "total time," including that spent at low temperature. A'', B'', and C'', hatching time of controls group kept at 28° C. A and B, hatching time at 28° C. of eggs exposed to 15° C. immediately after laying, and C of eggs exposed to 0° C. immediately after laying. A', B', and C', values for "total time" of the data in lines A, B, and C. For explanation see text.

In previous studies of the effect of temperature on the duration of diapause in grasshopper embryos it has been assumed that, if eggs hatched in a minimum time after exposure to low temperature, the diapause factors had been destroyed or inhibited by the low temperature. Unless embryos actually hatched before others with like potentialities kept at the high temperature, a critical situation does not exist. If the embryos hatched at the same time, whether part of that time was spent at a low temperature or all of it at a high temperature, the only conclusion allowable would be that temperature did not affect the breaking of diapause, since the process proceeded as satisfactorily at low temperature as at high—but no more so.

That both of these conditions exist after certain exposures to low temperatures in the material of the present investigation is evident from the data presented in Figures 3 and 5. The first situation is presented graphically by the lines C, C', and C'' and by the lines B, B', and B'' of Figure 3. It may indicate that diapause has been exhausted at the same rate both at high and low temperatures. The second condition is illustrated by the lines A, A', and A'' in Figure 3 and by curves similar to A of Figure 3 shown in Figure 5. It indicates that low temperature has actually shortened diapause or prevented its appearance.

While the average total time of similar populations subjected to high and low temperatures is of value in the analysis of the problem, it is not entirely adequate to give the clearest analysis of the effect of low temperature. The average may represent the central tendency in a single homogeneous population, or a balance between two or more central tendencies in a nonhomogeneous population. Figure 4 has been presented to

show that the average value for hatching time after exposures to low temperature may be a balance between two such central tendencies, one being characterized by the presence of a shortened diapause, the other by no indication of diapause.

Since the potential hatching time of the eggs when kept at 28° C. affects the response shown after exposure to low temperature, it is necessary in studying the effects of temperature to perform experiments on eggs having similar potentialities. The most extensive series of experiments which meet this requirement were those performed on the long diapause eggs collected during the autumn of 1935. The experiments were planned to study the effects of (1) incubation at 28° C. before exposure to low temperature (morphological stage), (2) different low temperatures, and (3) different periods of exposure to low temperature. Experiments performed on eggs of similar morphological stage are presented together.

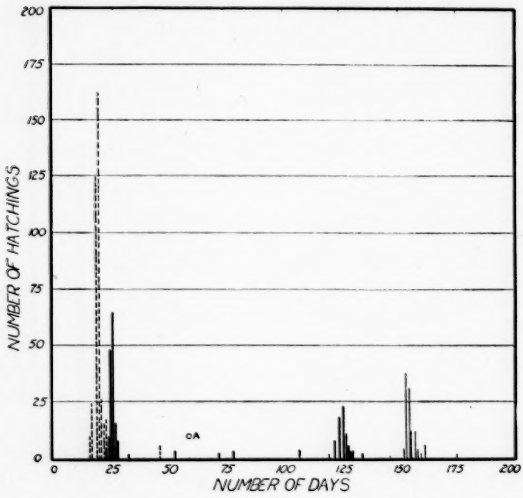

FIG. 4.—Showing distribution of hatching time at 28° C. in three samples of 500 eggs from the same population subjected to different conditions. Fine solid lines, control; heavy solid lines (two modes), non-incubated eggs exposed 28 days to 15° C.; *oA*, average of this sample; dashed lines, non-incubated eggs exposed 84 days to 15° C.

a) Eggs exposed to low temperatures immediately after laying (first day or non-incubated eggs).—Several types of response were shown by these very early eggs. The upper left-hand quadrant of Figure 5 gives the average hatching time at 28° C. of these non-incubated eggs. After any exposure to 0° and 5° C. the time required for hatching at 28° C. was reduced below that of the corresponding control groups by about the period of exposure to low temperatures, and the eggs hatched at the same time as those kept at high temperature. Examination of the data presented in section A of Table 4 shows that the percentage hatching of samples exposed to 0° and 5° was reduced below that of the control groups.

When these non-incubated eggs were exposed to 10° and 15° C., the average hatching time at 28° C. was reduced by more than the length of exposure to low temperature, and the eggs hatched before those in the corresponding control groups. Examination of the detailed data on hatching time in selected samples (Fig. 4) indicates that this result was produced by a balance between two responses. Some eggs had a reduction of time required at 28° C. equal to the period of exposure to low temperatures; others a much greater reduction to the minimum developmental time for the embryos. As the length of exposure approached 84 days, more of the eggs exhibited the maximum reduction. Those eggs exposed to 10° and 15° C. for long periods showed a marked increase in percentage hatching over control groups (Table 5).

b) *Eggs exposed to low temperatures after 7 days' incubation at 28° C. (comparable to 9 days at 25° C.).*—The responses shown by embryos which had developed for 7 days at 28° C. before exposure to low temperature differed in certain respects from those exhibited by younger eggs. The upper right-hand quadrant of Figure 5 presents the average hatching time at 28° C. of these eggs. No experiments have been performed at 0° C. comparable with the series performed at the other low temperatures; therefore no direct indication of the response to this temperature can be presented. The responses obtained after eggs had been exposed to the three low temperatures used were qualitatively alike.

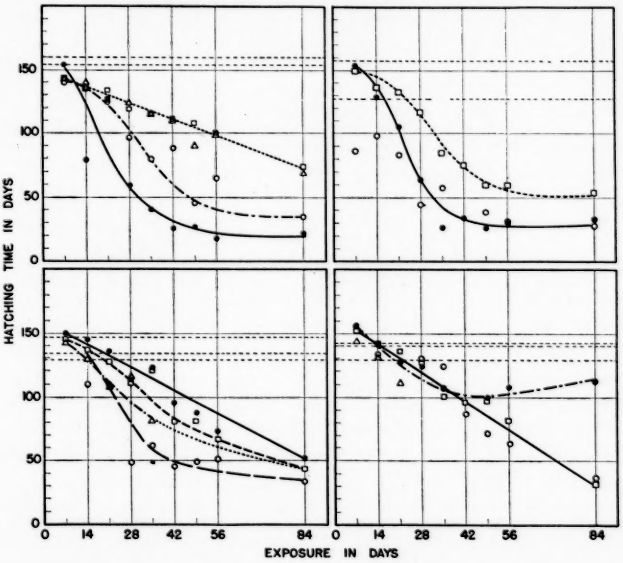

FIG. 5.—Data showing the effect of exposing eggs to low temperatures on the time required at 28° C. for hatching to occur. Upper quadrants, left to right, first-day eggs and seventh-day eggs; lower quadrants, left to right, fourteenth-day eggs and twenty-first-day eggs. Exposed to: dots—15° C., circles—10° C., squares—5° C., triangles—0° C. Dashed lines across the top are values of control groups involved. Each point is the average from a sample of 500 eggs.

Except in the case of 7-day exposures, the hatching time was reduced by more than the period of exposure to low temperature. Examination of the distribution of hatching time within each of the samples reveals that the average value for the group was the result of a balance between those eggs in which the hatching time was reduced by the period of exposure and those in which it was reduced to a minimum. This is comparable to the situation shown in Figure 4. One difference between eggs of this age and first-day eggs was that none of the conditions to which these were exposed reduced the percentage hatching. After some development had occurred, the eggs withstood exposures to 0° and 5° C. without suffering a reduction in percentage hatching, as was indicated by Bodine (1925). An increase in percentage hatching over control groups was present after long exposures to all three low temperatures. This is shown in Table 5. Table 4 compares

the percentage hatching of eggs exposed to 5° C. on the first day (col. A) and seventh day (col. B).

c) *Eggs exposed to low temperatures after 14 days' incubation at 28° C. (comparable to 19 days at 25° C.).*—The lower left-hand quadrant of Figure 5 presents the average hatching

TABLE 4*

SHOWING PERCENTAGE HATCHING OF CONTROLS AND EXPERIMENTAL GROUPS EXPOSED TO 5° C.

Period of Exposure (Days)	A		B	
	Percentage Hatching after Exposure to 5° C.	Percentage Hatching in Control Groups	Percentage Hatching after Exposure to 5° C.	Percentage Hatching in Control Groups
35	16.8	24.0	17.6	24.8
42	12.6	24.2	35.2	35.0
49	6.4	24.0	40.8	35.0
56	9.8	24.2	38.8	35.0
84	1.4	24.2	62.2	35.0

* First-day (col. A) and seventh-day (col. B) eggs exposed to 5° C. for various periods. Five hundred eggs in each experiment. Four populations involved. (Results obtained at 0° C. were similar.)

TABLE 5*

SHOWING DIFFERENCE IN PERCENTAGE HATCHING BETWEEN CONTROL GROUPS EXPOSED TO 28° C. AND LONG DIAPAUSE EGGS EXPOSED FOR 84 DAYS TO LOW TEMPERATURES

Days of Incubation before Exposure to Low Temperature	Low Temperature to Which Exposed	Difference between Experimental Groups and Controls (in Percentage)	Days of Incubation before Exposure to Low Temperature	Low Temperature to Which Exposed	Difference between Experimental Groups and Controls (in Percentage)
0	0	−20	14	5	72
0	5	−23	14	10	64
0	10	49	14	15	18
0	15	58	21	5	43
7	5	27	21	10	40
7	10	25	21	15	4
7	15	60			

* Each value derived from two samples of 500 eggs from the same mixture. Nine different mixtures, all showing similar characteristics, involved in the entire series.

time at 28° C. for the experiments with these eggs. The response to 0°, 5°, and 10° C. was qualitatively similar to that shown by 7-day-old eggs. This response was produced as in the preceding experiments by a balance between those eggs having hatching time reduced by approximately the period of exposure and those having it reduced to a minimum. The response of eggs exposed to 15° C. differed from those exposed to lower temperatures. Very short exposures increased the time required at 28° C., while longer exposures reduced it by approximately the period of exposure. The percentage of eggs

hatching was not reduced below the control groups by any exposures tried with 14-day-old eggs. An increase in percentage hatching was found after long exposures to all low temperatures used, as is shown by Table 5.

d) *Eggs exposed to low temperatures after 21 days' incubation at 28° C.* (*embryos in diapause*).—The lower right-hand quadrant of Figure 5 presents the average hatching time at 28° C. of the eggs in these experiments. Eggs exposed to 10° C. responded similarly to 14-day-old eggs. However, the reduction in time required at 28° C. after short exposures was almost negligible. Responses of eggs exposed to 5° and 15° C. were comparable after short exposures. They were similar in that both required increased time at 28° C. With longer exposures the responses at these two temperatures diverged. The embryos exposed to 5° C. showed reduction in the hatching time comparable to the response of eggs exposed to 10° C. Those exposed to 15° C. showed a slight indication of such reduction which tended to disappear after long exposures. It appears that long exposures of diapause eggs to 15° C. produced a secondary retardation in development which gave the appearance that the low temperature had not affected the hatching time. The percentage hatching was not decreased by any exposure in this series of experiments, and it was increased after long exposures, except at 15° C., as shown by Table 5.

DISCUSSION

Marked variations in the duration of diapause among the embryos of *M. differentialis* kept at constant high temperatures have been observed in the present investigation. There are differences within individual pods, among pods laid during different periods of the season, and among pods laid in various seasons. The reasons for these variations are still obscure.

Nothing is known about the genetic characteristics of the animals involved, except that no conscious selection was exercised in choosing them. They were all "wild" and probably very heterozygous. Very little is known about the environmental history of the animals, except that the summer of 1934 was extremely dry and hot while 1935 was wetter and much cooler, and the animals raised in the laboratory were at a more constant temperature, which had a higher mean value than either natural season. The conditions obtaining in 1934 and in the laboratory were more nearly alike than any other combination. It is interesting to note that the eggs collected in 1934 and in the laboratory were more nearly alike than any other combination, and that eggs from animals raised in the laboratory had the shortest diapause while those collected in 1935 had the longest.

The correlation of variations in length of diapause with time and the nature of the variations suggest that some environmental factors affect the appearance and duration of diapause. There is some resemblance in this material to the results reported by Steinberg and Kamensky (1936) for the effects of nutrition on the appearance and duration of diapause in *Loxostege sticticalis* or those reported by Watanabe (1924—abstracted, 1932–33) for the effect of temperature during larval stages on the voltinism of eggs of *Bombyx mori*.

The responses shown by the embryos of *M. differentialis* to periods of exposure to low temperatures are quite variable. The variations, however, are not random. They are conditioned by four factors: (1) the potentialities of the eggs in respect to length of diapause; (2) the amount of incubation before exposure to low temperature; (3) the low temperature to which they are exposed; and (4) the duration of the exposure to low temperature. A quantitative statement of the response can be formulated only for one

combination of the foregoing factors at a time. Qualitative descriptions of the responses can be more inclusive.

Some embryos exposed to low temperatures hatched before those left at a constant high temperature. To obtain this response the period of exposure must be shorter than the potential duration of diapause. The response indicates an actual effect of temperature on the processes involved in the diapause mechanism.

Some embryos exposed to low temperatures hatched at the same time as those left at constant high temperatures. This response might be the result of any one of three reactions. The time required at high temperature might be reduced by the period of exposure to low temperature when that is less than the length of the potential diapause of the eggs; the period of exposure might be equal to the duration of diapause of the material in an experiment properly belonging in the first category; or, by a coincidence, the proportion of individuals showing a loss of diapause might be such as to balance exactly a group showing no change in diapause condition so that the resulting average total time might be the same as for the control groups. Such a condition could be recognized by examining the distribution of hatching in the group. When the period of exposure equals the duration of diapause, a critical situation does not exist, and no conclusion can be formed as to how the response was elicited. In the present investigation all exposures of long diapause eggs have been of shorter duration than the diapause of the eggs.

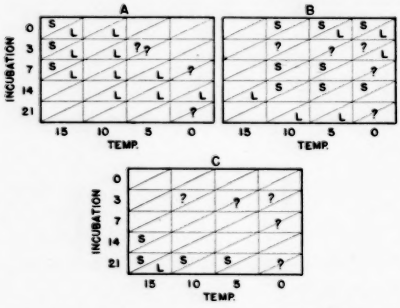

FIG. 6.—Summary, for long diapause eggs, of combinations of incubation, temperature, and exposure giving similar responses. *A*, those hatching before control groups; *B*, hatching at same time as control groups; *C*, hatching after control groups; *S*, exposures of 7–21 days; *L*, exposures of 28–84 days.

Some embryos exposed to low temperature hatched after those kept at a constant high temperature. This response might be due to any one of three reactions. The potentialities of the embryo might be maintained at the end of the exposure in the same condition as before, when the eggs should hatch as much later than the controls as the period of exposure to low temperature; the embryo might be injured by the exposure to low temperature, in which case no prediction of the result could be made; or the period of exposure to low temperature might be greater than the potential diapause of the material, in which case the total time of exposed eggs should be as much greater than that of control groups as the exposure exceeds the diapause.

The accompanying chart (Fig. 6) summarizes the responses of long diapause eggs to various combinations of the three variables—age, temperature, and duration of exposure. Question marks indicate situations for which no data are available. Similar charts might be constructed for short diapause eggs. However, in the present investigation, not enough experiments have been performed under conditions that yield critical results to warrant such charts. The few significant data show only the reduction of hatching time by the period of exposure to low temperature and exposed eggs hatching at the same time as control groups.

The distribution of the combinations of age, temperature, and period of exposure which produce each of the three responses suggest, in connection with data presented on the effects of temperature on developmental processes, that the ability of the embryo to accomplish some development while exposed to low temperature is in some way connected with the effectiveness of the combination in modifying the diapause of the embryo. Thus, considerable development occurred when non-incubated eggs were exposed to 15° C., while less occurred at lower temperatures. It is these same combinations at 15° C. which are most effective in shortening the diapause of the embryos, while exposures to 10° C. are more effective than to lower temperatures. If one knew nothing about the potentialities of eggs, but wanted to be sure to get large numbers of nymphs in the shortest possible time, the present investigation indicates that the optimum procedure would be to expose the eggs to 15° C. for about 6 weeks immediately after laying, then transfer to 28° C. until hatching occurs. With this procedure a total of about 9–10 weeks should elapse until the hatching of at least 80 per cent of the eggs.

Townsend (1926), in his study of hibernation in the codling moth, concluded that there is some process essential for the initiation of the next stage which proceeds well in dormant larvae only at low temperatures. It appears to go on most rapidly at 10° C. He suggests that the process was an enzymatic hydrolysis of larval tissues essential for the transformation to the pupal state. The similarity of results obtained by Townsend and in the present investigation suggest that here, too, some process essential for the initiation of the next stage (blastokinesis) goes on most rapidly at low temperatures, the optimum being 15° C. with this material. The evidence also suggests that the process is accelerated if the embryo itself is able to undergo development at a reduced rate. This effect of slow embryological development may be only in conditioning the vitality of the embryo when it is in condition to effect blastokinesis. The methods of the investigation do not furnish evidence on this point. Careful studies on metabolic activities of embryos after such experimental procedures might yield some information.

CONCLUSIONS

1. Eggs of the grasshopper *M. differentialis* show marked variations in duration of diapause when kept at 28° C. These variations are not random. Their nature suggests that they are determined by some as yet unknown environmental or physiological factors acting before the eggs are laid.

2. Eggs exposed to 15° C. immediately after laying will develop to a stage just before blastokinesis, but have never been observed to go beyond this.

3. The responses shown by these eggs after exposures to low temperatures fall into three categories: (*a*) the eggs hatch before controls; (*b*) the eggs hatch at the same time as controls; and (*c*) the eggs hatch after controls.

4. The response obtained depends upon four factors. These are: (*a*) potential diapause of the eggs, (*b*) amount of incubation when subjected to low temperature, (*c*) low temperature to which they are exposed, and (*d*) the duration of the exposure to low temperature.

5. Similarities between the results obtained in the present investigation and those obtained by Townsend with codling-moth larvae after exposure to low temperature are pointed out.

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THE EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON SURVIVAL AND METAMORPHOSIS OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)

(Four figures)

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BERGER (1907) showed that the meal worm, *Tenebrio molitor*, can be kept in an absolutely dry medium, and, in spite of a great loss of water, the relative water content of its body remains approximately constant. It is able to maintain a constant ratio of body water by the conservation of metabolic water (Mellanby, 1932). In contrast to the meal worm, Japanese beetle larvae are unable to conserve body water, and when desiccated at approximately 30 per cent relative humidity, all of the weight lost is due to a loss of water (Ludwig, 1936). This observation may be correlated with the fact that normally the beetle larvae are never exposed to the drying effects of the atmosphere since they live in moist soil. Furthermore, field observations indicate that these larvae are not able to survive desiccation, but that a high humidity is necessary for their development. Hence, it was thought desirable to study the effects of different relative humidities on the various developmental stages of the Japanese beetle. This study involves a consideration of the following: first, the rate of weight and water loss; second, the time of survival; third, the water content at death; and, fourth, the ability to metamorphose normally.

MATERIAL AND METHODS

Third instar larvae were collected in the field as needed. Prepupae and pupae were obtained from these larvae. Each individual was kept in a 1-ounce metal salve box containing moist plant mold. The humidity of the plant mold was kept near the point of saturation by the addition of tap water. Each larva was also given several grains of wheat to serve as additional food. Throughout the feeding period an abundance of food was always available, except during the period of experimentation, when the larvae were starved.

In this experiment, third instar larvae, early prepupae, and early pupae were used. The early prepupa differs from the third instar larva only in the creamy-white color of its body, the change in color being due to the elimination of fecal material. Each experiment was performed on a group of ten to twelve individuals. To obtain comparable results, prepupae, recognized as such 1–4 days previously, and pupae, 1 or 2 days after ecdysis, were used.

Each individual was weighed on a chainomatic balance, sensitive to $\frac{1}{10}$ mg., and placed in an unstoppered vial which was then kept at the proper humidity. The temperature was controlled near 25° C. Daily weight readings were made until death or metamorphosis occurred, when residual water content was determined by complete desiccation at a temperature of 50°–60° C. It was found that if the beetles are dehydrated at this

temperature for 2 days a constant weighing mass is obtained. Fecal material or exuviae were included in all weight readings.

The humidities were obtained by using saturated solutions of various salts in the bases of ordinary desiccators. The relative humidity values for these solutions, given in Table 1, are calculated from the data of Sweetman (1933) and the *International Critical Tables*.

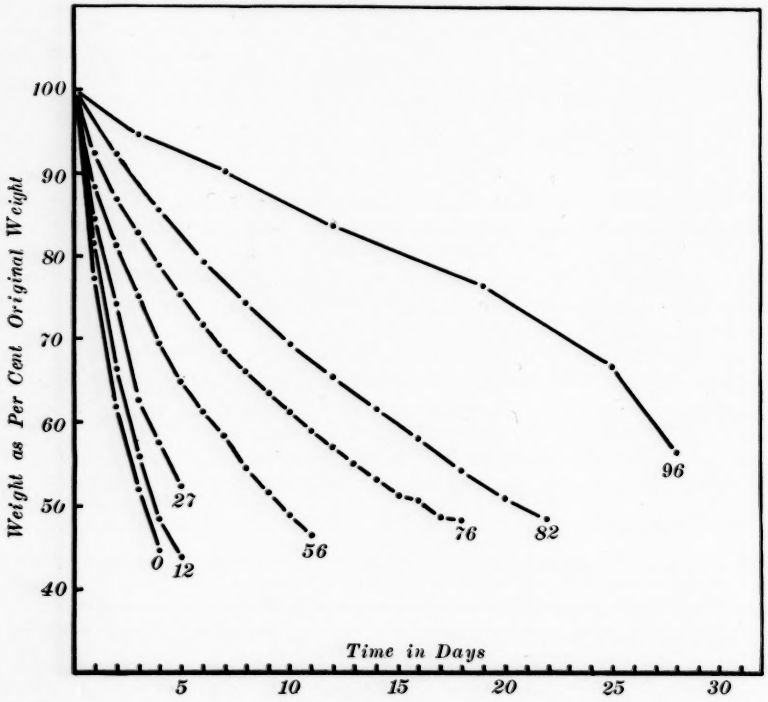

FIG. 1.—Rate of weight loss of third instar larvae at different relative humidities. Numbers indicate relative humidities.

OBSERVATIONS

The results obtained with third instar larvae, prepupae, and pupae are given in Figures 1–3 and in Table 1. In each figure the curves represent the averages of the individuals studied in each series. Likewise, the results contained in the table are average values.

Figure 1 shows that the rate of weight loss of third instar larvae is inversely proportional to the relative humidity, i.e., the lower the humidity, the greater the weight loss per unit time. Similar results were obtained for prepupae and pupae. A comparison of the rates of weight loss in the three stages at the relative humidities of 0, 56, 76, and 96 per cent is given in Figure 2. At all humidities used, except 96 per cent, the third instar

larva loses weight most rapidly. In each case, the curves are similar in form (except at 96 per cent), showing a more rapid weight loss at the beginning of the desiccation period followed by a progressive decrease in rate of weight loss. At 96 per cent relative humidity, weight loss occurs very slowly and is probably due to the utilization of body materials rather than a loss of water.

In Figure 3 the calculated water content is plotted for each stage at the relative humidities of 0, 56, 76, and 96 per cent. The third instar larva contains 81 per cent, the early

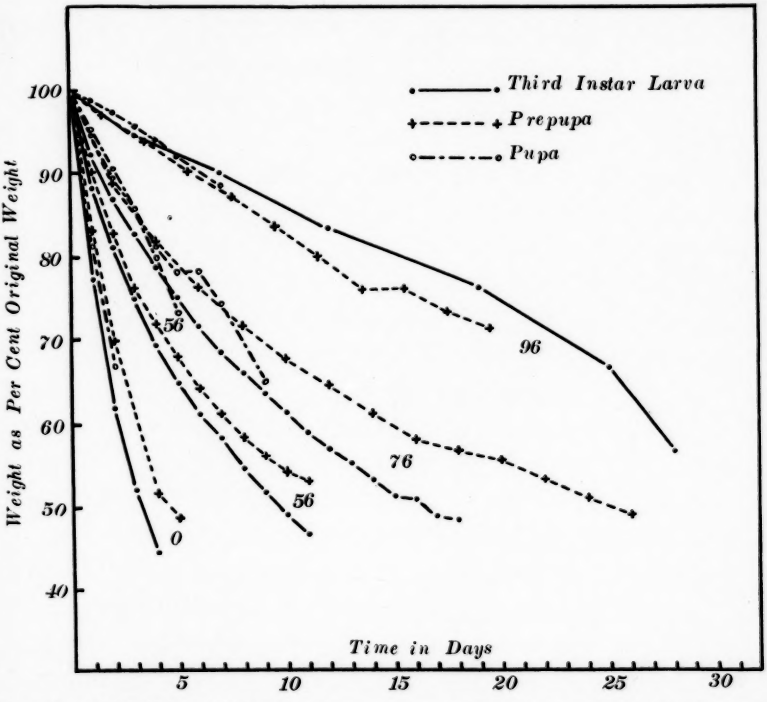

FIG. 2.—Rate of weight loss of third instar larvae, prepupae, and pupae at different relative humidities. Numbers indicate relative humidities.

prepupa 77.1 per cent, and the early pupa 76.5 per cent water (Ludwig, 1936). Using these figures as normal, the values plotted were obtained by assuming that all the weight lost was due to a loss of water. These calculated values are probably the same as the actual values as will be shown later.

Table 1 contains the calculated and observed water contents at death, and the length of time each stage survived at each humidity. An examination of this table shows that for each stage at each humidity, except 96 per cent, there is a very close agreement between the observed and calculated water contents at the time of death. In the case of third instar larvae, at each humidity except 96 per cent, regardless of the rates of weight

and water loss, death occurred when the individuals had desiccated to approximately the same degree (54.5–58.9 per cent water). At 96 per cent the individuals died when their water content was very much higher. This difference may be explained by the assumption that here death was due to starvation and not desiccation. Table 1 shows that in third instar larvae death by starvation occurred in 30.4 days at this humidity. The difference between the calculated and observed water contents at the time of death at 96

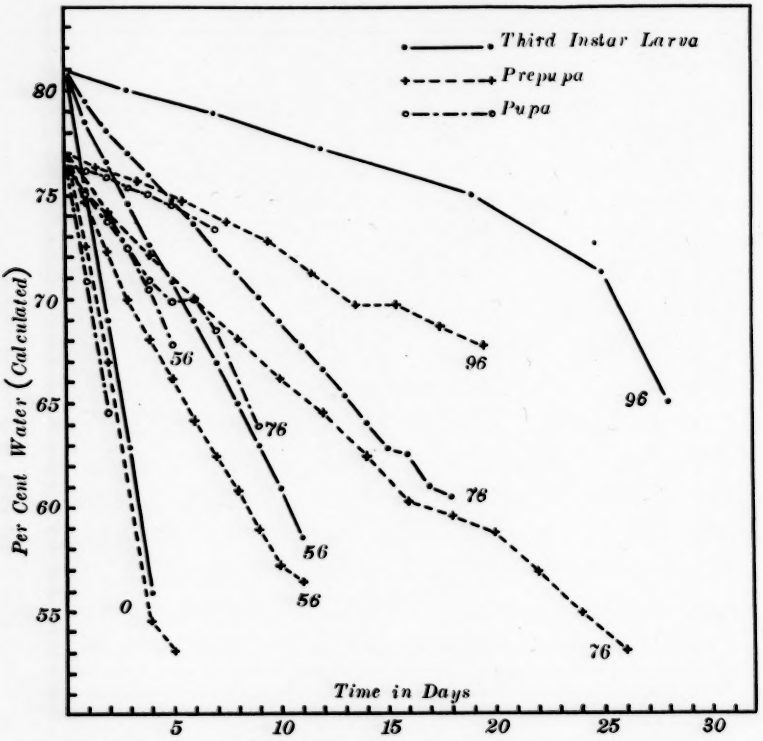

FIG. 3.—Rate of water loss of larvae, prepupae, and pupae at different relative humidities. Numbers indicate relative humidities.

per cent also indicates that a large amount of body material was utilized. The time of survival of third instar larvae varied directly with humidity, from 3.8 days at 0 per cent to 30.4 days at 96 per cent.

As indicated in Table 1, the prepupae can survive a desiccation to approximately the same degree (47.4–51.7 per cent water) regardless of the rate of desiccation, except at the humidities of 82 and 96 per cent. The time of survival of prepupae varied directly with the relative humidity up to 76 per cent, the observed values being 4.5 days at 0 per cent and 23.2 days at 76 per cent. At 82 and 96 per cent the figures given for time of survival

are not comparable to those obtained at other humidities, because at the higher values many of the individuals metamorphosed to the pupal stage. The duration of the pre-

TABLE 1

WATER CONTENT AND TIME OF SURVIVAL OF JAPANESE BEETLES STARVED AT DIFFERENT RELATIVE HUMIDITIES (NORMAL WATER CONTENT OF LARVAE =81.0 PER CENT; PREPUPAE, 77.1 PER CENT; PUPAE, 76.5 PER CENT)

Salt Used; Saturated Solution	Approximate Humidity (Per Cent)	No. of Individuals	Water Content at Death		Days Survived
			Calculated (Per Cent)	Observed (Per Cent)	
	Third Instar Larvae				
H_2O	96	11	59.1	68.2	30.4
$ZnSO_4$	82	12	58.2	54.5	23.2
NaCl	76	11	59.0	58.2	18.8
NaBr	56	10	54.5	56.0	12.5
$KC_2H_3O_2$	27	12	59.3	58.9	5.3
LiCl	12	12	56.0	57.7	4.6
$CaCl_2$ (solid)	0–5	12	55.8	57.7	3.8
Average			57.4	58.7	
	Early Prepupae				
H_2O	96	10	64.2	66.6	22.0
$ZnSO_4$	82	12	55.7	55.2	21.3
NaCl	76	10	53.6	51.7	23.2
NaBr	56	10	54.0	49.8	11.8
$KC_2H_3O_2$	27	10	53.0	48.9	6.8
LiCl	12	10	52.6	47.4	4.2
$CaCl_2$ (solid)	0–5	11	51.5	49.4	4.5
Average			55.3	52.9	
	Early Pupae				
H_2O	96	10	72.9	73.5	10.0
$ZnSO_4$	82	11	64.0	66.4	10.4
NaCl	76	10	64.9	67.5	7.3
NaBr	56	10	67.4	66.8	5.5
$KC_2H_3O_2$	27	12	68.3	67.4	2.9
LiCl	12	12	65.7	64.6	2.3
$CaCl_2$ (solid)	0–5	12	63.2	65.2	2.0
Average			66.6	67.3	

pupal period in these individuals is averaged along with the time of survival of the other forms. Correspondingly, the calculated and observed water contents are not comparable

to those found at other humidities since they include values obtained on individuals which molted to the pupal stage.

Table 1 also shows that pupae can survive desiccation to the same degree (64.6–67.5 per cent water) regardless of the rate of water loss, except at 96 per cent relative humidity. The time of survival varied directly with the humidity up to 82 per cent, the observed figures being 2 days at 0 per cent and 10.4 days at 82 per cent. Adult beetles emerged at humidities of 76 per cent and above, as shown in Table 2. At the higher humidities the time of survival and the calculated and observed water contents at death include the values obtained for those individuals which metamorphosed. At 96 per cent practically no weight or water loss occurred, and all but one individual metamorphosed. Hence in this case the days survived actually indicate the duration of the pupal period under the conditions of the experiment.

TABLE 2

RELATIVE HUMIDITIES AT WHICH METAMORPHOSIS OCCURRED

Metamorphosis	Relative Humidity (Per Cent)	Percentage of Individuals Metamorphosing
Larva to prepupa	96	0.0
	82	16.7
	76	0.0
	56	0.0
Prepupa to pupa	96	75.0
	82	8.3
	76	0.0
	56	0.0
Pupa to adult	96	90.0
	82	54.5
	76	33.3
	56	0.0

Table 2 gives the relative humidities at which metamorphosis occurred. If third instar larvae are taken from the field and starved at different relative humidities, metamorphosis is not possible, the larvae dying of starvation at the higher values. In this experiment, 16.7 per cent of the larvae began metamorphosis at 82 per cent. However, these larvae had been kept at room temperature for about a month before the start of the experiment, and metamorphosis was observed approximately 10 days later. Unlike the third instar larva the prepupa and pupa are non-feeding stags; hence, in these forms the ability to metamorphose is directly dependent, among other factors, on the humidity of the environment. Metamorphosis of prepupae to pupae occurred at 83 and 96 per cent, and of pupae to adults at 76, 82, and 96 per cent. In each case more individuals metamorphosed at the higher humidities.

As previously stated, for each stage there is a very close agreement between the calculated and observed water contents at death. Hence, practically all weight loss is water loss and there is no conservation of metabolic water in third instar larvae, prepupae, or pupae.

DISCUSSION

The average observed water contents at the time of death, regardless of humidity, were 58.7 per cent for third instar larvae, 52.9 per cent for prepupae, and 67.3 per cent for pupae (Table 1). These figures are in agreement with those of Ludwig (1936), who found that the maximum limits of desiccation at which survival was possible varied as follows: third instar larva, 62.0 per cent; early prepupa, 59.1 per cent; and early pupa, 65.9 per cent.

Ludwig (1936) found that there is no conservation of metabolic water in the third instar larva of the Japanese beetle. In the present experiments it was also found that the prepupal and pupal stages are not able to conserve water. This is probably associated with the fact that all three stages live in moist soil where they are normally never exposed to low humidities. Evans (1935) studied the influence of humidity on the loss of weight of prepupae and pupae of the blowfly, *Lucilia sericata*, and found that the greater loss of weight at low humidities is due to a loss of water and not of dry matter. Similar results were obtained by MacLeod (1935), who states that the tick, *Ixodes ricinus*, is not able to utilize metabolic water, the loss of weight being a direct indication of the rate of evaporation of water from the body.

The fact that the rate of evaporation of water from a free surface is approximately proportional to the saturation deficiency of the atmosphere is known as "Dalton's law." It has been found to apply to the loss of water from the bodies of certain insects by a number of investigators. Among these studies the following may be cited: Bacot and Martin (1924) exposed adults of the rat flea, *Xenopsylla cheopis*, to four different humidities at 32° C., and found that the duration of life was directly determined by saturation deficiency except in saturated air; Mellanby (1932) used the larvae of *T. molitor* and observed that the loss of water was directly proportional to saturation deficiency; Buxton (1932) plotted data obtained from the work of Bodine (1921) for the hibernating nymphs of *Chortophaga viridifasciata*, and showed that the relation between loss of water and saturation deficiency was linear. Figure 4 shows the relationship between saturation deficiency and the loss of water from the third instar larva, prepupa, and pupa of the Japanese beetle. The rate of water loss in each stage is directly proportional to the saturation deficiency, and hence Dalton's law has been found to apply to these forms.

The time of survival of larvae, prepupae, and pupae varied directly with the humidity, except at the higher values, where metamorphosis occurred or the individuals died of starvation. These results are in agreement with those of Bodenheimer (1930) on the duration of life of starved individuals of the desert locust, *Schistocerca gregaria*. He found that at 0 per cent relative humidity they lived only 2.9 days, while at 100 per cent they survived 4.5 days. On the other hand, Leeson (1932) found that 90 per cent was optimum for unfed rat fleas; the duration of life being 3.7 days at 0 per cent, 7.3 days at 90 per cent, and 6.5 days at 95 per cent. MacLeod (1935) showed that in the tick, *I. ricinus*, 70 per cent relative humidity was optimum for survival.

The ability of the Japanese beetle to metamorphose is limited to high humidities, prepupae being able to transform to pupae at 82 per cent and above, and pupae to adults at 76 per cent and above. In both cases a higher percentage of metamorphoses occurred at the higher humidities. These observations agree with those of Beck (1932), who observed that in the fly, *Rhagoletis suavis*, the greater the relative humidity, temperature remaining constant, the greater the adult emergence. On the other hand, Griswold and

Crowell (1936) found that in the webbing clothes moth, *Tineola bisselliella*, 75 per cent relative humidity proved to be optimum for development and metamorphosis.

The fact that high humidities are necessary for the metamorphosis of the Japanese beetle undoubtedly serves as an important environmental factor limiting its distribution.

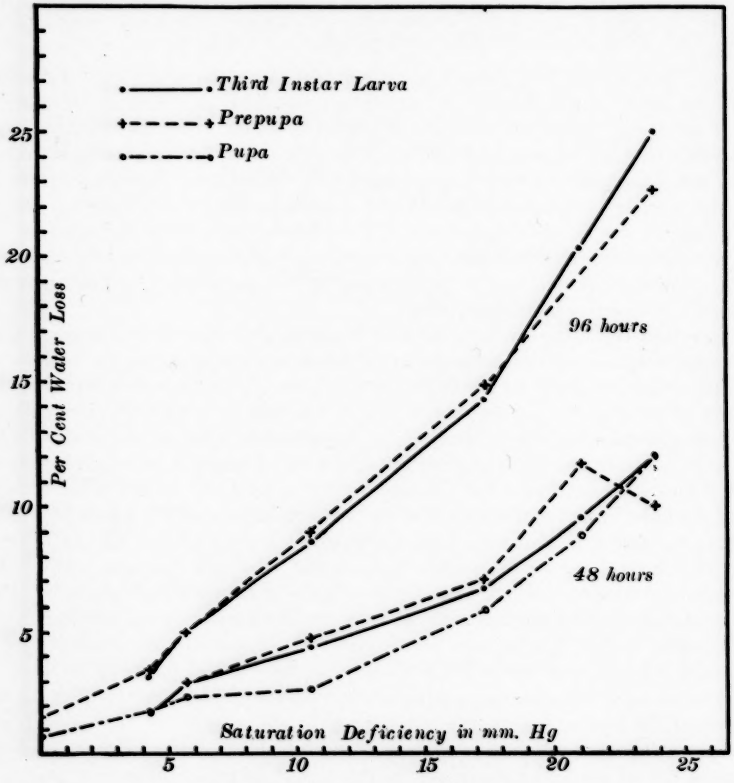

FIG. 4.—Rate of water loss at different saturation deficiencies. Lower group of curves represent percentage of water loss during first 48 hours; upper group during first 96 hours.

It will not be able to survive if introduced into the arid or semi-arid regions of the country. Not only is a high humidity necessary for metamorphosis, but Fox (1934) reported some observations which indicate that it is also necessary for embryonic development and hatching. He found that the beetle population in heavily infested areas was greatly reduced in 1933 as compared with 1932. He suggested that this reduction was probably associated with a deficient rainfall during the preceding summer at the time oviposition and hatching were at a maximum.

SUMMARY AND CONCLUSIONS

1. The loss in weight and water of third instar larvae, prepupae, and pupae of the Japanese beetle are inversely proportional to the relative humidity of the environment.

2. The time of survival of third instar larvae is greater at higher humidities. In the case of prepupae and pupae many individuals metamorphosed at the higher humidities, and therefore the time of survival (which includes the duration of the prepupal or pupal stages in the metamorphosing individuals) is reduced.

3. Loss in weight is due to water loss; hence, there is no conservation of metabolic water in the three stages studied.

4. Death occurred at approximately the same water content regardless of the rate of desiccation.

5. An almost saturated medium is necessary for the metamorphosis of prepupae and pupae, whereas third instar larvae are unable to metamorphose at any humidity when starved.

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THE EFFECTS OF CERTAIN ORGANIC ACIDS ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA[1]

(One figure)

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IN AN earlier paper (Rulon, 1936) data were presented which indicated that in *Euplanaria dorotocephala* the head frequency of animals, reconstituting from short transverse pieces cut from the posterior levels of the anterior zooid, was increased by subjecting the pieces during the reconstitutional period to slightly toxic solutions of carbon dioxide and the H-ion. It was likewise found that the head-frequency gradient could be altered by treating the animals with certain concentrations of carbon dioxide for several days before section. Calcium in the form of calcium chloride was found to antagonize the injurious effects of the H-ion over a limited pH range but failed to antagonize the actions of carbon dioxide. The H-ion had little or no effect between pH values of 7 and 4.4, but between 4.3 and 4.15 it altered the head frequency while causing only a limited number of deaths.

Since the strong acid (HCl) used in making up the H-ion solutions ionizes almost completely, it was concluded that the H-ion as such did not penetrate the tissue of the reconstituting animal below a certain concentration (pH=4.3). The question of the possible effects on head frequency of certain weak organic acids which ionize to only a limited degree was at once suggested by these results. It is well known that different organic acids penetrate living cells, probably in molecular form, quite easily, and in doing so may affect certain physical and physiological properties of the living protoplasm.

THE HEAD-FREQUENCY GRADIENT IN "EUPLANARIA"

Child (1911) noted that short transverse pieces of *E. dorotocephala* reconstituted differently when taken from different regions of the body axis. Short pieces from just posterior to the head usually reconstituted into animals with normal heads while pieces of the same size from the posterior regions of the first zooid usually gave rise to headless individuals or those with a markedly inhibited type of head. Child also noted that the types of heads which appeared showed a graded inhibition from the normal through a series of inhibited heads to a headless form. In later work Child (1911*a*, 1911*b*, 1911*c*, 1912, 1914*a*, 1914*b*, 1916, 1920, 1924) has classified the different forms reconstituting in *Euplanaria* into five classes based on the degree of inhibition of the head. Briefly the types are:

1. *Normal.*—Head triangular with two symmetrically placed eyes and auricles at lateral margins.

2. *Teratophthalmic.*—Head normal in shape and auricles in normal position but with eyes showing all degrees of approximation to the median plane from two distinct eyes with pigment connected to complete cyclopia.

[1] Support for this work was provided from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

3. *Teratomorphic.*—Head more or less rounded in outline with single, or apparently single, median eye; auricles more or less anterior and showing all degrees of approximation to the median plane to a single median auricle.

4. *Anophthalmic.*—Heads rudimentary without eyes and with or without a single median auricle.

5. *Acephalic.*—Head completely absent.

Child and Watanabe (1935) point out that the inhibition of heads arising at the anterior cut surfaces of short pieces of *Euplanaria* is brought about by some inhibiting factor originating at the posterior cut surface. This head-inhibiting factor is more than the mere stimulus of section, for it persists, as shown by the delay of anterior section, for several days during the early stages of tail growth (Child and Watanabe, 1935). Inhibited heads do not appear in animals reconstituting from long pieces and from short pieces from anterior levels. Watanabe (1935) shows a gradient in rate of head formation decreasing posteriorly in this species, which suggests that short transverse pieces from anterior regions may, because of a higher physiological activity, be able to overcome the posterior inhibiting factor while like pieces from posterior regions of the zooid, because of a lower physiological activity, are unable to do so. Experiments by Child and Watanabe (1935) show that short transverse pieces from any level of the body can be induced to form a normal head if posterior section is delayed for a few hours. Apparently, after 6–8 hours the head-forming cells become activated sufficiently to form a normal head regardless of any posterior inhibiting factor which may then be imposed.

Various workers have found that certain agents in the reconstituting medium will increase the head frequency in short transverse pieces of *Euplanaria*. Such agents are dilute KCN (Child, 1916), chloretone, chloroform, chloral hydrate, ether, ethyl alcohol (Buchanan, 1922), and caffeine (Hinrichs, 1924). Mechanical stimulation (Child, 1920) and increases in temperature (Behre, 1918) will also increase head frequency. It has also been found that head frequency can be decreased in pieces from anterior levels and long pieces with such agents as metabolic end products, ethyl alcohol, dilute KCN (Child, 1911*a*, 1912, 1916), anesthetics (Buchanan, 1922), and caffeine (Hinrichs, 1924) with certain exposure times and concentrations of the solutions. Behre (1918) reported that a sudden decrease in temperature would lower the head frequency in reconstituting pieces.

It seems to be quite clear that agents which increase the head frequency do so either by stimulating the head-forming cells of the anterior cut surface or by blocking the head-inhibiting action of the posterior cut surface. A decrease in head frequency is apparently brought about by a decrease in the activity of the head-forming cells or an increase in effectiveness of the posterior inhibiting factor.

MATERIAL AND METHODS

The material used in this investigation was the flatworm *E. dorotocephala*, which is found in large numbers in fresh-water springs to the south and west of Chicago. After collection the stocks were brought to the laboratory and kept in well water in large granite pans for several weeks before using in the experiments. In the laboratory the water was changed on at least alternate days. The animals used for experimentation were always starved for approximately 2 weeks before section. In this work only animals 14–16 mm. in length were used.

All the animals were sectioned as shown in Figure 1. Only the anterior zooid was used,

and the transverse pieces from each level were all approximately one-eighth the length of the entire animal, exclusive of the head. Sixty-five animals were sectioned for each lot. Of this number of pieces from each level fifty of the most nearly equal in size were selected. This procedure eliminated much of the error which may have been introduced by faulty cutting. Equal numbers of pieces from animals of the same stock were used for tests and controls. The animals were always washed and sectioned in the solutions in which they were allowed to reconstitute.

After section the pieces from each level were placed in the test or control solutions in tightly stoppered 1-liter Erlenmeyer flasks. The solutions were changed daily at the beginning of this investigation, but as time went on it was found that much more constant results could be obtained if the flasks containing the pieces were placed in the dark soon after the experiment was begun and not molested until the time for the observations. The reason for this soon became evident in that the very act of changing the solutions may stimulate the pieces to motor activity—a factor which in itself will cause the different levels to have an increased head frequency (Child, 1920). Because of the great volume of solution compared to the size of the pieces and the fact that the type of head is determined by at least 2 or 3 days after section, this experimental procedure appears to be justified.

1

FIG. 1.—Levels *A*, *B*, *C*, and *D* used in all head-frequency experiments. Each section is approximately one-eighth the length of the animal, exclusive of the head. Head and region posterior to the mouth were discarded.

Two weeks after section the reconstituted animals were observed with a binocular microscope and the types of heads recorded. A fairly reliable method for evaluating the approximate average degree of head inhibition in pieces of a given size and from a particular level has been presented by Child and Watanabe (1935). This method consists of designating each type of head by an arbitrary number which corresponds roughly to the degree of inhibition. The arbitrary values of each type are as follows: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20. In any particular lot of worms reconstituted from pieces of a given level the number of each head type multiplied by its arbitrary number and the sum of all divided by the total number of living animals gives the head-frequency index (designated as "mean" by Child and Watanabe). It is readily seen that in animals reconstituting from *A* levels, which usually develop nearly all normal heads, the index will approach 100. On the other hand, animals reconstituting from *D* levels, which most often under control conditions give rise to headless forms, have an index approaching 20. Large numbers of separate experiments in this laboratory have shown that a comparison of head-frequency indices of test and control give a reliable method for discerning the effects of various agents on reconstitution.

In testing the effects of the various organic acids used in this investigation it was necessary in preparing the test solutions to use the well water in the laboratory since it was used throughout as the control solution. The addition of acid liberated large quantities of carbon dioxide in the well water since it was rich in bicarbonates. Carbonate-free well water was therefore prepared by a method similar to that described by

Smith and Clowes (1924) for the freeing of sea water of carbonates. Hyman (1925), following Smith and Clowes's procedure, prepared a carbonate-free well water by adding a sufficient quantity of HCl to break down all the bicarbonates and removing the carbon dioxide from the solution by bubbling air through it for at least 24 hours. In our experiments 4½ cc. of concentrated HCl was added to 16 liters of well water to insure complete breakdown of the bicarbonates. After the carbon dioxide was removed from the solution, M/10 NaOH was added until a pH of 7 was obtained. This neutral carbonate-free well water was used in making up the test solutions (it has been found in previous experiments that such water has little or no effect on the head frequency in *Euplanaria*).

Previous experiments had shown that it was necessary to use a concentration of the test reagent which was slightly below that producing cytolysis in intact animals. Solutions of graded concentrations were therefore made up and intact animals placed in each. After several attempts it was possible to choose a concentration which would produce cytolysis in only a limited percentage of the pieces exposed. The exact molarity of the different test solutions was determined by titration. In the earlier part of this work concentrations considered to be the most favorable were (*a*) formic acid: 0.00025 M; (*b*) acetic acid: 0.00036 M; (*c*) propionic acid: 0.00032 M; and (*d*) isobutyric acid: 0.000176 M. In additional experiments performed 18 months later on an entirely different stock of worms collected at a different locality at a different season it was found that these concentrations were toxic to such an extent that all pieces from all levels died within 24 hours. The effective concentrations used in the later work were (*a*) formic acid: 0.00015 M; (*b*) acetic acid: 0.00025 M; (*c*) propionic acid: 0.0002 M; and isobutyric acid: 0.0001 M.

EXPERIMENTAL DATA

THE EFFECT OF FORMIC ACID ON HEAD FREQUENCY AND VIABILITY

Since the previous experiments have shown that calcium protects pieces of *Euplanaria* in concentrations of the H-ion which would otherwise be effective, it was of interest to determine in these experiments if the effects of formic acid could be altered by increasing the calcium content of the solutions. Accordingly, in each set of experiments three lots of worms were used. The pieces of one lot were allowed to reconstitute in well water (pH=7.5) which served as a control. The test solutions were 0.00025 M formic acid and 0.00025 M formic acid plus 0.5 gm. of $CaCl_2$ (anhydrous) per liter. Experiments were performed on three different stocks of worms, and, although results of experiments on each stock were not identical, the relationship of the tests to the control of worms from each stock was the same. The data from the three different experiments are grouped together in Table 1.[2]

A comparison of the percentages of the different head types reconstituting in well water and in 0.00025 M formic acid indicates an increase in head frequency in the test material at *C* and *D* levels. The pieces were quite susceptible to this concentration of formic acid, but experiments with lower concentrations (0.0002 M) failed to show any definite effects on either head frequency or viability in these stocks. The head-frequency indices in these experiments should be considered with caution since the computation is made only from living animals. It is clear that in experiments where many of

[2] In all the tables the reconstituting animals are classified according to the inhibition of the head as follows: I, normal; II, teratophthalmic; III, teratomorphic; IV, anophthalmic; V, acephalic.

the pieces die the indices, particularly at *C* and *D* levels where the forms may range from normal or teratophthalmic to acephalic, may mean selective death. In general, a high death-rate may conceivably be due to a higher death frequency of the forms undergoing most complete reconstitution, for such forms are primarily more susceptible than anophthalmic and acephalic forms; on the other hand, it may mean that the pieces undergoing more complete reconstitution can tolerate the concentration used while those undergoing less reorganization cannot. In the first case, the death-rate in general decreases from *A* to *D* levels with the frequency of complete reconstitution; in the second, it increases from *A* to *D* levels with decrease in frequency of complete reconstitution. A comparison of the percentage of pieces dying at the different levels in formic acid suggests that the anterior levels are more susceptible than the posterior levels. Sixty-seven per cent of *A* pieces compared to 32 per cent of *D* pieces died during 2 weeks' exposure

TABLE 1

THE EFFECTS OF FORMIC ACID AND FORMIC ACID PLUS CALCIUM CHLORIDE ON RECONSTITUTION IN "EUPLANARIA" (150 ANIMALS USED IN EACH TEST AND CONTROL—VALUES GIVEN IN PERCENTAGE)

Solution Used	Level	I	II	III	IV	V	Dead	Index
Well water (control)	A	93	5	.....	.....	.....	1	99
	B	57	30	2	3	8	.....	85
	C	28	20	10	20	22	.....	63
	D	15	12	7	13	52	.....	47
Formic acid, 0.00025 M	A	32	1	.....	.....	.....	67	99
	B	38	8	.....	.....	1	54	94
	C	49	17	2	3	1	28	90
	D	25	13	4	13	13	32	68
Formic acid, 0.00025 M, +0.5 gm. $CaCl_2$ per liter	A	92	5	.....	.....	.....	3	99
	B	83	9	1	1	1	5	96
	C	29	37	2	19	13	1	70
	D	21	23	3	13	39	1	55

to the 0.00025 M formic acid. This indicates primary or direct lethal effect, not differential tolerance.

A comparison of the data from pieces which have reconstituted in 0.00025 M formic acid and 0.00025 M formic acid plus 0.5 gm. of $CaCl_2$ per liter (Table 1) is of interest. Calcium evidently has a protective action, as is shown by the decrease in number of pieces killed as well as a possible decrease in the head frequency. A further experiment dealing with the protective action of calcium was conducted using 0.00027 M formic acid and 0.00027 M formic acid plus 0.5 gm. of $CaCl_2$ per liter as test solutions. The effects of these solutions on pieces from different levels are shown in Table 2. It is readily seen that this concentration of formic acid is extremely toxic for pieces of *Euplanaria*. None of the pieces from levels *A* and *B* and only a few of the pieces from levels *C* and *D* survive when formic acid alone is used. With calcium, however, this concentration of formic acid is not nearly so toxic, as shown by a tremendous decrease in the number dying, although the head-frequency indices remain considerably above those for animals reconstituting in well water.

THE EFFECT OF ACETIC ACID ON HEAD FREQUENCY AND VIABILITY

As in the case of formic acid, each experiment dealing with the effects of acetic acid on reconstitution in *Euplanaria* involved three lots of animals from the same stock. The control lot reconstituted in well water (pH = 7.5) while the tests reconstituted in 0.00036 M acetic acid and in 0.00036 M acetic acid plus 0.5 gm. $CaCl_2$ per liter. This experiment

TABLE 2

THE PROTECTIVE ACTION OF CALCIUM ON "EUPLANARIA" RECONSTITUTING IN TOXIC CONCENTRATIONS OF FORMIC ACID (50 ANIMALS USED IN EACH TEST AND CONTROL)

Solution Used	Head-Frequency Index				Percentage Dead			
	A	B	C	D	A	B	C	D
Well water (control)	100	57	25	27	0	0	0	0
Formic acid, 0.00027 M	.....	.....	88	40	100	100	90	96
Formic acid, 0.00027 M, +0.5 gm. $CaCl_2$ per liter	100	97	70	69	88	38	4	44

TABLE 3

THE EFFECTS OF ACETIC ACID AND ACETIC ACID PLUS CALCIUM CHLORIDE ON RECONSTITUTION IN "EUPLANARIA" (50 ANIMALS USED IN EACH TEST AND CONTROL)

Solution Used	Expt.	Head-Frequency Index				Percentage Dead			
		A	B	C	D	A	B	C	D
Well water (control)	1	100	53	25	22	0	0	0	6
	2	100	86	58	45	0	0	0	0
	3	100	89	50	41	4	2	0	0
Acetic acid, 0.00036 M	1	100	70	28	21	4	0	44	40
	2	100	94	55	38	0	0	14	50
	3	100	93	41	27	2	4	2	42
Acetic acid, 0.00036 M, +0.5 gm. $CaCl_2$ per liter	1	100	60	29	24	0	0	0	48
	2	100	94	62	54	0	2	18	46
	3	100	92	50	33	4	2	12	24

was performed three different times on different stocks of animals. The head-frequency indices and the percentage of the animals dying by the end of the reconstitutional period are recorded in Table 3.

In these experiments it seems justifiable to compare only the head-frequency indices and percentage dying since the possibility of increased indices because of selective deaths appears to be absent. It will be noted that in the controls the different stocks show different head-frequency indices. The importance of the physiological condition of the animals at the time of cutting, particularly as regards head frequency, has been

discussed at some length in the earlier paper (Rulon, 1936) and will be considered later. A comparison of the controls and tests in 0.00036 M acetic acid suggests an increase in head frequency at *B* levels in the tests. On the other hand, the other levels indicate little if any effect of this reagent on the head frequency in animals from these stocks.

A comparison of the deaths of pieces in the test and control solutions shows definitely that this concentration of acetic acid is very near the lethal level. It will be noted that the highest percentage of deaths appears in pieces from the posterior levels while *A* and *B* levels are able to withstand almost completely the toxic actions of this concentration.

The data from the tests in which 0.00036 M acetic acid plus 0.5 gm. of $CaCl_2$ per liter was used are of interest. In the head-frequency experiments there is little evidence that the addition of calcium to acetic acid may determine head frequencies which are

TABLE 4

THE EFFECTS OF PROPIONIC ACID AND PROPIONIC ACID PLUS CALCIUM CHLORIDE ON RECONSTITUTION IN "EUPLANARIA" (100 ANIMALS USED IN EACH TEST AND CONTROL—VALUES GIVEN IN PERCENTAGE)

Solution Used	Level	I	II	III	IV	V	Dead	Index
Well water (control)	A	97	3	.....	.....	.....	.....	99
	B	17	22	1	20	40	.....	51
	C	.....	.....	.....	16	84	.....	23
	D	.....	.....	1	3	93	3	21
Propionic acid, 0.00032 M	A	75	.....	.....	.....	.....	25	100
	B	96	.....	.....	.....	.....	4	100
	C	25	9	1	5	9	51	75
	D	3	.....	.....	3	.....	94	70
Propionic acid, 0.00032 M, +0.5 gm. $CaCl_2$ per liter	A	97	.....	.....	.....	.....	3	100
	B	97	1	.....	1	.....	1	99
	C	36	15	.....	5	5	39	84
	D	15	3	.....	5	1	76	82

nearer to the control head frequencies than are those of animals which have reconstituted in acetic acid alone. Moreover, calcium seems to be very ineffective in protecting pieces in 0.00036 M acetic acid from death, as is shown by a comparison of the percentage dead in Table 3. Since the effects (if any) of acetic acid on head frequency are so slight compared to the effects of other agents, it seems that we are justified in concluding that the chief, and perhaps only, effect of acetic acid at this concentration on these stocks is to kill the animals and that this effect is not hindered to any appreciable extent by calcium.

THE EFFECTS OF PROPIONIC ACID ON HEAD FREQUENCY AND VIABILITY

The experiments with propionic acid have given the most interesting results of all the organic acids studied. The concentration found to be the most favorable for these stocks was 0.00032 M. As in the other experiments with organic acids, parallel experiments were performed to test the combined effects of calcium and the acid. In Table 4 are presented the combined data of two separate experiments in which 100 worms were used for each test and control. Comparison of animals which have reconstituted in well

water and those in 0.00032 M propionic acid shows clearly the effects of this agent. At *B* levels only 17 per cent of the controls give normal heads while 100 per cent of the living test animals which have reconstituted from pieces of this level are normal. Comparison of the living animals reconstituted from *C* and possibly *D* levels shows the same effect of propionic acid although the majority of the pieces from these levels died.

Equally interesting is the distribution of deaths according to the level from which the pieces were taken. The higher percentage of deaths at more posterior levels is no accident. Repeated experiments have shown that pieces from these levels cannot survive, for any extended period of time, the toxicity of this concentration. Later experiments show that pieces from *C* and *D* levels usually die only after an exposure of 3 or more days to the propionic acid solution. If the pieces from these levels are kept in propionic acid for 3 days and then returned to well water for the rest of the reconstituting period, they will usually survive and develop largely into animals with normal heads.

Comparison of pieces which have reconstituted in 0.00032 M propionic acid with pieces in the same concentration but with 0.5 gm. of $CaCl_2$ added per liter indicates a very slight protective action of calcium against death. This protective action is evidenced to some degree at all four levels. It is interesting to note that, with this protection by calcium from the lethal effects of propionic acid, *C* and *D* pieces show an increase in head frequency.

It has been noted that pieces from the more anterior levels often reconstitute a much overdeveloped head in this concentration of propionic acid. By an "overdeveloped" head it is meant that the head is definitely larger in comparison to the size of the piece than would be expected. It is distinctly broader and the eyespots are definitely farther apart than in normal heads reconstituting in well water. Since pieces from these levels are more susceptible than those from posterior levels and new tissue is more susceptible than old to lethal concentrations, this overdevelopment of the head in anterior pieces is undoubtedly also the result of differential tolerance. In other words, the highly active new tissue of anterior pieces is able to neutralize or otherwise dispose of the acid in the concentration used more rapidly or more completely than other parts.

THE EFFECTS OF ISOBUTYRIC ACID ON HEAD FREQUENCY AND VIABILITY

Three experiments involving three different stocks of worms were performed in which isobutyric acid was used. As in the case of the other organic acids, each experiment involved one control and two tests. The test solutions were 0.000176 M isobutyric acid and 0.000176 M isobutyric acid plus 0.5 gm. of $CaCl_2$ per liter.

The three different stocks behaved somewhat differently as regards head frequency and viability when the pieces reconstituted in isobutyric acid. From the data presented in Table 5, in which only the head-frequency indices and percentage dead are given, it will be noted that animals with low head-frequency indices in the controls (Exper. 1) were affected very little, if at all, by this concentration of isobutyric acid. Animals with higher head-frequency indices in the controls (Expers. 2 and 3) showed increased head-frequency indices in *B*, *C*, and *D* pieces. The value of these indices is uncertain since a large number of the pieces died and the apparent increase in head frequency may be the result of selective death. Other experiments with isobutyric acid, however, have shown that this agent actually does increase the head frequency (Table 7).

The data indicate that the test solutions with isobutyric acid plus calcium are at least as effective as the test solutions with isobutyric acid alone in increasing the head-

frequency indices and percentage of deaths. In fact, when all three experiments are considered, it appears that the test solutions with calcium are even more effective. At least it seems quite certain from these data that calcium exerts very little, if any, protective action against isobutyric acid.

HEAD FREQUENCY AND VIABILITY OF "EUPLANARIA" CONDITIONED TO CARBON DIOXIDE AND ALLOWED TO RECONSTITUTE IN PROPIONIC ACID SOLUTION FOR VARYING INTERVALS OF TIME

Of the four organic acids used in this investigation it has been found that propionic acid was by far the most effective in increasing the head frequency in the more posterior levels of the first zooid. Earlier experiments have shown that animals kept in high concentrations of carbon dioxide for several days before sectioning give an increased head frequency after section (Rulon, 1936). In this series of experiments carbon dioxide was

TABLE 5

THE EFFECTS OF ISOBUTYRIC ACID AND ISOBUTYRIC ACID PLUS CALCIUM CHLORIDE ON RECONSTITUTION IN "EUPLANARIA" (50 ANIMALS USED IN EACH TEST AND CONTROL)

Solution Used	Expt.	Head-Frequency Index				Percentage Dead			
		A	B	C	D	A	B	C	D
Well water (control)	1	100	66	25	22	0	0	0	2
	2	100	92	41	42	4	0	0	2
	3	100	78	37	24	6	2	6	0
Isobutyric acid, 0.000176 M.	1	100	66	31	23	0	0	2	0
	2	100	99	96	81	50	42	46	66
	3	100	98	73	62	8	30	40	76
Isobutyric acid, 0.000176 M, +0.5 gm. $CaCl_2$ per liter	1	100	77	29	34	0	0	0	44
	2	100	99	.....	95	34	14	100	78
	3	100	96	57	73	6	24	2	58

used before section and propionic acid after section in an attempt to determine how much the head frequency could actually be increased and its association with viability.

The experiments involved six lots of animals from the same stock. One lot was kept in well water throughout and served as a control. The other five lots were treated for 3 days in carbon dioxide solution (pH = 5.6) followed by 7–10 days in carbon dioxide solution (pH = 5.2). In Table 6 the conditioned animals are referred to as "Tests." They were sectioned and allowed to reconstitute in (I) well water, pH = 7.5; (II) 0.00032 M propionic acid for the first 24 hours; (III) 0.00032 M propionic acid for the first 72 hours; (IV) 0.00032 M propionic acid for the first 96 hours; and (V) 0.00032 M propionic acid for the entire reconstitutional period. After the initial exposure to propionic acid in Tests II, III, and IV the pieces were returned to well water.

The results of this double treatment are exceedingly interesting. In Test II it will be noted that there was a tremendous increase in head frequency at *B*, *C*, and *D* levels. With 24 hours' exposure to propionic acid *B* levels yielded 88 per cent normal heads, *C* levels 38 per cent normal heads, and *D* levels 50 per cent normal heads. In well water

similar animals gave 6 per cent normal heads at *B* levels, no normal heads at *C* levels, and 2 per cent normal heads at *D* levels. This increase in head frequency in propionic acid was accomplished without an increase in the death-rate. With an initial exposure to 0.00032 M propionic acid of 72 and 96 hours the head frequencies were even higher than with 24 hours' exposure. Comparison of the head-frequency indices shows that with this treatment the head frequency at *D* levels is not significantly different from the head frequency at *A* levels. From the data it is seen that up to 96 hours' exposure there is no increase in deaths.

TABLE 6

THE EFFECTS OF PROPIONIC ACID ON RECONSTITUTION IN CARBON DIOXIDE-CONDITIONED "EUPLANARIA" (50 ANIMALS USED IN EACH TEST AND CONTROL—VALUES GIVEN IN PERCENTAGE)

Solution Used	Level	I	II	III	IV	V	Dead	Index
Well water (control)	A	100	.....	.....	.....	.....	.....	100
	B	22	36	6	20	16	.....	66
	C	.....	.....	.....	26	74	.....	25
	D	.....	.....	.....	8	90	2	22
Well water, Test I	A	100	.....	.....	.....	.....	.....	100
	B	6	20	2	24	48	.....	42
	C	.....	2	.....	22	76	.....	26
	D	2	2	8	6	74	8	28
Propionic acid, 0.00032 M, 24 hr., Test II	A	100	.....	.....	.....	.....	.....	100
	B	88	8	.....	2	.....	2	97
	C	38	40	4	12	6	.....	78
	D	50	38	2	6	4	.....	85
Propionic acid, 0.00032 M, 72 hr., Test III	A	100	.....	.....	.....	.....	.....	100
	B	96	2	.....	.....	.....	2	100
	C	90	8	.....	.....	2	.....	97
	D	86	10	.....	.....	4	.....	95
Propionic acid, 0.00032 M, 96 hr., Test IV	A	100	.....	.....	.....	.....	.....	100
	B	100	.....	.....	.....	.....	.....	100
	C	92	6	.....	.....	.....	2	99
	D	86	10	2	.....	.....	2	97
Propionic acid, 0.00032 M, entire period, Test V	A	98	.....	.....	.....	.....	2	100
	B	94	.....	.....	2	.....	4	98
	C	74	6	.....	.....	.....	20	99
	D	14	6	.....	.....	.....	80	94

Test V, in which the worms were conditioned to carbon dioxide and the pieces from the different levels allowed to reconstitute in 0.00032 M propionic acid for the entire reconstitutional period (approximately 2 weeks), shows one interesting result. The head-frequency indices are almost the same as in Tests III and IV but the percentage dying at the different levels is vastly different. A gradient of deaths occurs. The greatest number dying (80 per cent) is at *D* level, with 20 per cent at *C* level, 4 per cent at *B* level, and 2 per cent at *A* level. These data point clearly to the fact that the posterior levels are less able than anterior levels to tolerate long-time exposure to certain agents.

THE EFFECTS OF THE DIFFERENT ORGANIC ACIDS ON RECONSTITUTION WITH AN INITIAL EXPOSURE OF 72 HOURS

It has been pointed out in the preceding experiments that certain of the organic acids in the concentrations used were toxic to the extent that a large number of the pieces died during the course of the experiment. It has also been pointed out that a large number of deaths may obscure the results in such a way that the head frequency, particularly at the more posterior levels, may appear increased although actually the apparent increase may be accounted for, at least in part, by selective death.

It has been noted in the experiments, with propionic acid (Table 6) that pieces from posterior levels died only after several days' exposure. Much work in this laboratory has shown that in short transverse pieces the type of head is usually determined by at least 3 days after section. Accordingly it was decided to test the effects of the different organic acids on head frequency by using an initial exposure of 72 hours to the acid, after which the pieces were allowed to finish their reconstitution in well water. Parallel experiments were performed in which pieces were maintained in like solutions for the entire reconstitutional period.

It has been previously stated that this later series of experiments was performed some 18 months after those described in the preceding sections. The animals had been collected at a different locality and in a different season. This stock was considerably more susceptible to the various concentrations of the organic acids used than the previous stocks. It was therefore necessary to use the lower concentrations of the acids referred to on page 183.

In Table 7 are recorded the data for 500 animals—100 reconstituting in well water and 100 in each of the organic acids. Of these, 50 were allowed to reconstitute in the test solution for 72 hours followed by return to well water, and 50 were kept in the test solution for the entire reconstitutional period.

In the case of formic acid it is clearly shown by a comparison with the control that the head frequency is increased in pieces from *B*, *C*, and *D* levels. This increase is apparent with both 3-day and full-time exposure to the agent. It appears as if *A* pieces may show a slight decrease in head frequency with continued exposure, but the data are inadequate to permit definite conclusions. In this stock, with the concentration used, few deaths occurred even with long-time exposure.

Contrary to the preceding experiments, pieces reconstituting in acetic acid show a distinct increase in head frequency at *C* and *D* levels when compared with the control. With long-time exposure most of the *C* and all the *D* pieces died while all *A* and *B* pieces lived. This shows quite definitely a failure on the part of the posterior levels to tolerate this agent.

Although a number of pieces died in propionic acid, it is clearly shown that the head frequency is considerably increased over the controls. With long-time exposure the failure of the posterior levels to tolerate this agent is also clearly shown.

In isobutyric acid the head frequency is evidently increased although many pieces died with the 72 hours' exposure. Death frequency is highest in *A*, less in *B* and *C*, and lowest in *D* pieces and therefore indicates direct lethal effect, not differential tolerance. Although a greater percentage of pieces died at *C* and *D* levels with long-time exposure than with 72 hours' exposure, it is believed that the differences as shown in this experiment alone are not sufficiently great to warrant any definite conclusions as regards lack of tolerance of pieces from these levels to this agent.

DISCUSSION

With the experimental data at hand it is now possible to group the four organic acids used according to their toxicity as follows: isobutyric > formic > propionic > acetic.

TABLE 7

THE EFFECTS OF THE DIFFERENT ORGANIC ACIDS ON RECONSTITUTION WITH AN INITIAL EXPOSURE OF 72 HOURS (50 ANIMALS USED IN EACH TEST AND CONTROL—VALUES GIVEN IN PERCENTAGE)

Solution Used	Classes	Solution Changed to Well Water after 72 Hours				Initial Solution Used throughout Reconstituting Period			
		A	B	C	D	A	B	C	D
Well water (control)	I	90	36	.....	4	94	50	12	2
	II	10	36	2	.....	6	30	4	2
	III	.....	.....	2	.....	.....	4	6	4
	IV	.....	6	10	4	.....	2	10	12
	V	.....	20	86	88	.....	10	68	78
	Dead	.....	2	.....	4	.....	4	.....	2
Index		98	73	24	24	99	83	36	27
Formic acid, 0.00015 M	I	86	52	32	46	74	54	40	32
	II	8	40	10	8	20	34	16	20
	III	2	.....	6	2	.....	.....	4	8
	IV	.....	4	8	12	2	2	10	8
	V	.....	4	36	30	2	6	26	32
	Dead	4	.....	8	2	2	4	4	.....
Index		98	86	59	66	93	87	67	62
Acetic acid, 0.00025 M	I	86	76	42	32	94	84	14	.....
	II	10	12	6	8	4	12	4	.....
	III	.....	4	4	.....	2	.....	.....	.....
	IV	.....	2	18	14	.....	.....	6	.....
	V	.....	6	26	42	.....	4	4	.....
	Dead	4	.....	4	4	.....	.....	72	100
Index		98	90	64	55	98	94	73	.....
Propionic acid, 0.0002 M	I	88	86	80	68	84	72	.....	2
	II	.....	2	4	6	.....	2	.....	.....
	III	.....	.....	.....	.....	.....	.....	.....	.....
	IV	.....	.....	.....	6	.....	.....	.....	.....
	V	.....	.....	2	2	.....	.....	.....	.....
	Dead	12	12	14	18	16	26	100	98
Index		100	99	97	92	100	99	.....	100
Isobutyric acid, 0.0001 M	I	58	46	34	26	64	42	32	18
	II	4	8	6	16	4	14	16	4
	III	.....	4	4	2	.....	2	2	.....
	IV	.....	2	6	8	.....	8	4	12
	V	.....	10	20	44	.....	14	2	50
	Dead	38	30	30	4	32	20	44	16
Index		99	82	68	54	99	76	85	43

With the exception of formic acid the toxicity increases with the length of the carbon chain. This grouping has been arranged on the basis of the molarity of the acid solutions

rather than on the basis of the pH values. The approximate H-ion concentrations of the different organic acids used in the earlier experiments were found with La Motte color indicators to be: isobutyric—4.9, propionic—4.8, acetic—4.7, and formic—4.2.[3] Previous work has shown that the H-ion, as such, has little effect on reconstitution or viability in *Euplanaria* between pH 7.0 and 4.4 but that between pH 4.3 and 4.15 it is quite effective in altering the head frequency and increasing the death-rate. From this it would seem that the effectiveness of isobutyric, propionic, and acetic acids is not directly due to the H-ion concentration. With formic acid a somewhat different conclusion must be drawn. The H-ion is present in a concentration that does affect reconstitution. This fact suggests that the effectiveness of formic acid in increasing head frequency and producing death may be related to the ionization constant of the acid aside from the length of the carbon chain.

Child and Watanabe (1935) and Watanabe (1935*b*) have given evidence of a transmission of the factor preventing head formation from its posterior point of origin to the head-forming region at least in part by way of the ventral nerve cords. This factor prevents in some way the dedifferentiation and activation of the head-forming cells presumably by maintaining them as functional parts of the body through nervous stimulation. It therefore seems logical to assume that agents which penetrate the tissue may block the excitations arising at the posterior cut surface by their actions on the nervous elements transmitting this factor. If this is the case, any inhibiting agent which has a greater effect on nervous tissue than on other tissues of the piece will be likely to cause an increase in head frequency.

It has been noted that the different organic acids behave differently at slightly sublethal concentrations. Pieces surviving and reconstituting in the proper concentrations of propionic acid will develop an extremely high percentage of normal heads (Tables 4, 6, and 7). Acetic acid, on the other hand, while causing a definite increase in head frequency of posterior pieces from certain susceptible stocks (Table 7) fails to cause an increase in other less susceptible stocks (Table 3). Formic and isobutyric acids caused an increase in the head frequency in the different stocks but apparently not to the order of the increase caused by propionic acid. Of course it may be argued that the concentrations of acetic, formic, and isobutyric acids were not sufficient to cause such an increase in the head frequency as propionic. Higher concentrations for the different stocks, however, produced death in most or all the pieces from all levels. It may be suggested that the organic acids behave differently in altering head frequency by a differential action on the nervous tissues. In other words, certain acids affect the nervous system to a greater extent and bring about a more effective block to the posteror inhibitory action.

The distribution of deaths at the different levels is of interest as an aspect of the longitudinal gradient. With acetic, propionic, and possibly isobutyric acids the greatest numbers of pieces dying during the course of reconstitution are from posterior levels when the proper concentrations are used. With acetic and propionic acids (Tables 6 and 7) the posterior pieces die after 3 or 4 days while the anterior pieces are largely able to tolerate the toxic effects of the solution the entire reconstitutional period.

[3] If the pH values are calculated from the normalities and the ionization constants of the different acids, it will be found that the H-ion concentrations observed here are too low. If we had been working with distilled water, however, in place of carbonate-free well water, no doubt the observed pH would have been much closer to the calculated values.

Several factors may be concerned in determining this distribution and the time of death. As Child has shown in various papers, higher levels of the gradient are more susceptible than lower levels to concentrations above the limit of tolerance, but are also more able than lower levels to tolerate a certain range of lower concentrations and to acquire increased tolerance, that is, to acclimate in the course of time. Moreover, susceptibility is temporarily increased following section to a greater degree in pieces from more posterior than in those from more anterior levels. And, finally, susceptibility to concentrations above the limit of tolerance and ability to tolerate and to acquire increased tolerance to lower concentrations increase in the course of reconstitution.

In the earlier experiments with formic acid more pieces from the anterior levels died during the course of the experiment than did pieces from posterior levels (Tables 1 and 2). In fact, there seemed to be some evidence of a gradation of deaths in an anteroposterior direction. This differential is similar to that observed with other agents in lethal concentrations. The later experiments with formic acid (Table 7) cannot be considered since the concentration used was not sufficient to produce deaths at any level. Formic acid behaves differently than the other organic acids studied in that the H-ion concentration of the formic acid solution used (Tables 1 and 2) is so high that this acid is undoubtedly effective through its H-ion and perhaps also in its molecular form. The data indicate that the distribution of deaths of pieces reconstituting in formic acid is a direct expression of differential susceptibility not involving differential tolerance to any appreciable degree. The anterior pieces die early because their susceptibility is primarily high, while pieces from the posterior levels die later because their susceptibility is primarily low; and they are killed more slowly than anterior pieces perhaps only as their susceptibility increases in the course of reconstitution. It is evident from the data, however, that in general pieces from the more anterior levels tolerate the concentrations of acetic and propionic acids used to a greater degree than those from the more posterior levels. With some stocks this is also true with isobutyric acid (Table 5) but with other more susceptible stocks the data are less clear (Table 7). Whether this difference results from a difference in the limit of tolerance with difference in the original gradient level or from a difference in ability to acquire increased tolerance—that is, to acclimate or become conditioned to the acids—remains for further experiment to determine.

Mention must again be made of the relation between physiological condition of the animals at the time of section and head frequency. It has been noticed throughout this work that animals giving a low head frequency in pieces from *C* and *D* levels in the controls usually show less increase in head frequency under the experimental conditions used than those with a higher head frequency at these levels. This is shown in the experiments with isobutyric acid (Table 5). Evidently the different stocks used in the isobutyric acid experiments show different head frequencies because of different environmental conditions prior to section. The experiments in which animals were conditioned with carbon dioxide and pieces from these animals allowed to reconstitute in propionic acid (Table 6) demonstrate clearly how certain internal factors may be inhibited or obliterated if external environment before and during reconstitution is controlled. Differences in physiological condition of the stocks of animals may also determine differences in death frequency at the different levels. Likewise, some stocks of animals may be more susceptible to certain concentrations of organic acid than other stocks (Table 7) and may react to the test reagents differently. This is clearly shown in the case of experi-

ments with acetic acid which failed to cause an increase in head frequency in the less susceptible stocks (Table 3) but caused a definite increase in head frequency in more susceptible stocks (Table 7).

Earlier work has shown that previous environment is important in determining the steepness of the head-frequency gradient (Rulon, 1936). In general it appears that animals in good condition show a steeper head-frequency gradient, that is, head development is inhibited to a greater degree at the more posterior levels of the anterior zooid than in animals in poor condition. The head-frequency gradient is almost invariably steeper in newly collected animals than in stocks kept for a long period in the laboratory with laboratory feeding. In fact, stocks in which disintegration is occurring in the head regions of many animals usually show a high head frequency at the posterior levels. The experiments with the different acids suggest that, when the factor inhibiting head development is highly effective, a more intense action of the external agent is necessary to decrease it to the threshold of ineffectiveness than when it is less effective. If this inhibiting factor is wholly or in a large part nervous in character, as the experiments of Watanabe (1935*b*) indicate, the differences in head frequency resulting from differences in physiological and external condition must be associated with the physiological condition of the nervous system.

The protective action of calcium against certain ions is well known, and there is some evidence that calcium may protect living tissues from certain molecular effects. Loeb (1915) and Armstrong (1928) have demonstrated that $CaCl_2$ will prevent to some extent the penetration of acetic acid into the living *Fundulus* embryo. In the experiments on reconstitution of pieces of *Euplanaria* in organic acids it has been found that calcium antagonizes very little, if at all, the effects of acetic acid in producing death. It is entirely possible, however, that short-time exposure to high concentrations such as Loeb and Armstrong used would show the antagonistic action of calcium, while long-time exposure to a weak solution would fail to do so. With isobutyric acid it was found that calcium failed to alter, at least in any regular manner, the effects of the agent on either viability or head frequency. With propionic acid head frequency was increased while the percentage of deaths was lowered when calcium was added to the test solution. With formic acid, however, the protective action of calcium is clearly shown. Not only is the percentage of deaths decreased through the addition of calcium to the test solution, but the increase in head frequency caused by formic acid is at least partially prevented (Tables 1 and 2). But in the case of formic acid solutions the H-ion is an important factor. It therefore appears from these experiments that calcium, while clearly protecting living tissues from certain effects of the H-ion as the experiments with inorganic acids also show (Rulon, 1936), fails to prevent to any great extent the penetration into planarian tissues or organic acids which are present partly in undissociated form.[4]

SUMMARY

1. Anterior zooids of *E. dorotocephala* (14–16 mm. in length) were cut into four transverse pieces of equal size. Each piece was approximately one-eighth the length of the animal. The head was excluded. Pieces from each of the four levels were allowed to reconstitute in solutions of formic, acetic, propionic, and isobutyric acids in concentrations which usually produced death in a number of the pieces. In most cases parallel experi-

[4] The author is indebted to Professor C. M. Child for much helpful advice and criticism received throughout this work.

ments were performed in which 0.5 gm. of $CaCl_2$ were added to each liter of test solution. Equal numbers of pieces were allowed to reconstitute under identical conditions in well water as controls. Test solutions were made up with carbonate-free well water.

2. In certain stocks 0.00025 M formic acid caused an increase in the head frequency of animals reconstituting from the posterior levels. In like stocks the death frequency decreased from anterior to posterior levels. Deaths and increase in head frequency were decreased by increasing the calcium concentration of the solution. In more susceptible stocks the head frequency was increased although viability was not affected by either 72-hour or full-time exposure to 0.00015 M formic acid.

3. Acetic acid (0.00036 M) did not alter the head-frequency gradient in any definite manner in certain less susceptible stocks but did produce a death frequency which increased from anteroir to posterior levels. These effects were not prevented by calcium. Seventy-two-hour and full-time exposure to 0.00025 M acetic acid caused an increase in head frequency in the posterior levels of animals from the more susceptible stock—the data showing that pieces from the posterior levels die only after as much as 72 hours' exposure to the agent.

4. Propionic acid (0.00032 M) caused a tremendous increase in the head frequency of pieces from *B*, *C*, and *D* levels of animals from the less susceptible stocks. The greatest number of deaths was from *C* and *D* levels. Calcium added to the acid caused a slight decrease in the number of pieces dying during the course of the experiments and an increase in the head frequency in the pieces from *C* and *D* levels. In the more susceptible stocks 0.0002 M propionic acid was equally effective—the pieces from posterior levels dying chiefly after 72 hours' exposure to the agent.

5. Although certain experiments indicated an increase in head frequency at posterior levels in the less susceptible stocks with 0.000176 M iosbutyric acid, the data were not conclusive. A postero-anterior gradation of deaths was indicated in certain experiments. In the more susceptible stocks 0.0001 M isobutyric acid caused a definite increase in head frequency at the posterior levels.

6. When a certain stock was conditioned to carbon dioxide, sectioned, and allowed to reconstitute in 0.00032 M propionic acid for 72–96 hours and then returned to well water, it was found that all levels gave almost 100 per cent normal or near-normal heads. Few, if any, pieces died with as much as 96 hours' exposure to the test agent. When the carbon dioxide–conditioned pieces were kept in the test solution for the entire reconstitutional period, a distinct gradation in death frequency decreasing from posterior to anterior levels occurred.

7. The decrease in death frequency in formic acid from anterior to posterior levels parallels the differential susceptibility of intact animals to agents in concentrations above the limit of tolerance. The decrease from posterior to anterior levels in the other acids indicates a differential tolerance like that observed in intact animals subjected to a certain range of lower concentrations of various agents.

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STUDIES IN POPULATION PHYSIOLOGY. VII. THE RELATION OF ENVIRONMENTAL CONDITIONING TO THE DECLINE OF TRIBOLIUM CONFUSUM POPULATIONS

(Three figures)

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PREVIOUS investigations (Park, 1934, 1935, 1936) have analyzed certain effects of conditioned flour upon the fecundity and fertility of the beetle, *Tribolium confusum* Duval. "Conditioned flour" represents a medium which is produced by the beetles in the normal course of their existence and is obtained, for experimental purposes, by using flour which has been inhabited by dense populations of *Tribolium*. It is, therefore, an inevitable product of population activity which owes its existence entirely to the cumulative effects of the breeding and general behavior of the beetles themselves. More specifically, conditioned flour differs from fresh or unconditioned flour in that the beetles inhabiting it have reduced its nutritive value through constant feeding and have added certain by-products, such as excretory and metabolic wastes, not present in the original medium. It was shown in one of these previous reports (Park, 1934) that, if the fecundity of *Tribolium* females was assayed while the forms were living in a heavily conditioned flour and contrasted with the fecundity of a control or fresh-flour group, the former beetles had a drastically lower rate of egg production than the latter.

In a later paper (Park, 1936) the heavily conditioned flour (i.e., flour from a dense *Tribolium* population) was "diluted" with fresh flour so as to form a series of mediums composed of 25, 50, and 75 per cent conditioned flour. The effects of these environments on the beetles' fecundity were tested against a fresh-flour control and an all or heavily conditioned flour control in order to see if differential conditioning of the environment—i.e., a lighter or partial conditioning—was equally effective in changing oviposition rates.

The results indicated that, in general, there was a proportionate relationship between the degree of conditioning and the number of eggs produced. In other words, the beetles in the fresh flour laid the most eggs; those in the 25 per cent conditioned flour and the 50 per cent conditioned flour laid eggs at the next highest rate; those in the 75 per cent followed; and the forms in all-conditioned or "undiluted" mediums produced the fewest eggs. This was not a perfectly proportional relationship; the greatest difference came between the fresh-flour controls and the 25 and 50 per cent series. (The beetles in the latter two environments produced eggs at a statistically similar rate.) In other words, there was a bigger difference, as far as fecundity was concerned, between the fresh-flour controls and those living in the most lightly conditioned medium (the 25 per cent) than there was between any of the other combinations. This immediately suggested that it might be instructive to investigate the influence of more lightly conditioned flours upon *Tribolium* productivity and to try to relate these facts to the data previously obtained. The emphasis of the present paper is directed toward this end with the special hope that certain generalizations about the decline of *Tribolium* populations will be forthcoming.

The ultimate interest behind all these conditioned-flour experiments has been not so much in the description of certain physiological facts—although these are of certain intrinsic value—as in the attempt to relate these facts to a more generalized picture of the dynamics of *Tribolium* populations. This was, of course, the emphasis of Chapman (1928), who first used *Tribolium* in experimental population studies, and it has been the emphasis of later workers as well.

REMARKS ON TECHNIQUE

The technique of rearing and handling *T. confusum* has been described in several places, and it is not necessary to repeat it in detail at this point. It should be mentioned, however, that these beetles and their immature stages spend the entire life-cycle in flour and that their eggs can be obtained for counting by sifting the populated flour through fine silk bolting cloth. The beetles of the present experiments were members of the same inbred stock used in previous investigations. The experiments were run at 28° C., and at a relative humidity of about 40 per cent. The fresh flour was an unbleached, patent flour known as "Ceresota," and the heavily conditioned flour was taken from cultures of *Tribolium* which had passed their maximum size. Thirty-two grams of flour in a half-pint milk bottle containing two young (2–3 weeks old) virgin *Tribolium* males and two such females constituted a single experimental unit for all the experiments. The only factor deliberately varied was the type of flour. An extended description of the life-cycle and general biology of this beetle has been published by Park (1934*a*) and may be consulted for further information. Good (1936) has also prepared recently a paper on this subject.

In preparing the mediums used in the present experiments heavily conditioned flour was added to fresh flour in the proportions necessary to make up environments in which the conditioned-flour content was 5, 10, 15, 20, and 25 per cent. It is obvious that in combining materials such as flour great precautions must be taken to assure a thorough and homogeneous mixture of the two elements. This was done by mechanically sifting the newly mixed flours a number of times and then carefully stirring by hand the resulting medium. For each of the environments thus prepared enough flour was mixed to supply all the experimental bottles without further mixing, i.e., all the bottles were supplied from one source, and the same was true for each of the other groups. This minimized the possibility of a flour difference between bottles of any one series.

EXPERIMENTATION

In the first experiment populations were started to test the effect of slight flour conditioning upon the fecundity of *T. confusum* over 5-day intervals for a period of 40 days. The experiment was set up so that there were 70 bottles, each containing 32 gm. of fresh flour; 25 bottles, each containing 32 gm. of heavily conditioned flour; 45 bottles containing 5 per cent conditioned flour; 45 bottles containing 10 per cent conditioned flour; 45 bottles containing 15 per cent conditioned flour; 45 bottles containing 20 per cent conditioned flour; and 45 bottles containing 25 per cent conditioned flour. Of the fresh-flour bottles, 45 had their medium renewed at each 5-day count and are designated in this report as the "fresh" series. In the other 25 fresh-flour bottles the flour was not changed during the entire 40-day period, and these are designated the "fresh conditioned" series. The medium was not renewed in any of the conditioned-flour groups at the time of egg-counting, although flour lost through handling was replaced to keep the total

quantity up to 32 gm. This replacement flour was taken from extra cultures comparable to the various experimental series and run expressly for the purpose of making such additions.

TABLE 1

MEAN EGG PRODUCTION PER BOTTLE PER 5-DAY READINGS

Flour	Days							
	5	10	15	20	25	30	35	40
Fresh (renewed)	27.2±1.45	21.0±0.86	25.0±0.93	29.2±0.72	31.7±0.94	29.5±0.81	26.5±0.87	28.6±0.91
Fresh conditioned	19.0±1.59	17.3±1.42	16.9±1.24	20.7±1.67	21.4±1.44	19.8±1.01	20.0±1.22	18.7±0.86
Heavily conditioned	12.5±1.34	9.6±0.86	6.3±0.63	9.3±1.11	7.4±0.78	7.3±0.62	7.8±0.63	7.3±0.53
5 per cent conditioned	27.2±1.14	20.3±0.72	16.4±0.58	17.8±0.89	16.5±0.73	16.1±0.78	16.3±0.71	15.9±0.70
10 per cent conditioned	26.9±1.05	20.2±0.78	17.4±0.64	16.9±0.59	16.1±0.68	15.3±0.74	15.0±0.71	15.5±0.57
15 per cent conditioned	26.4±1.18	22.9±0.71	18.7±0.56	16.0±0.58	16.0±0.70	16.4±0.76	14.4±0.52	14.5±0.63
20 per cent conditioned	25.0±1.05	21.0±0.88	16.1±0.51	15.0±0.71	13.0±0.60	13.8±0.59	14.2±0.54	13.5±0.55
25 per cent conditioned	25.9±0.97	19.8±0.75	14.5±0.59	14.7±0.50	12.8±0.47	12.6±0.45	11.8±0.34	14.0±0.35

Each bottle was initially seeded with two virgin male and two virgin female beetles all from the same stock culture and of similar age. The procedure was to introduce the adult *Tribolium* into their respective bottles, allow them to oviposit for 5 days, pass the flour through the bolting-cloth sieve (thereby collecting the eggs for counting), and then return the beetles to their environment for 5 more days of egg-laying. Every effort was

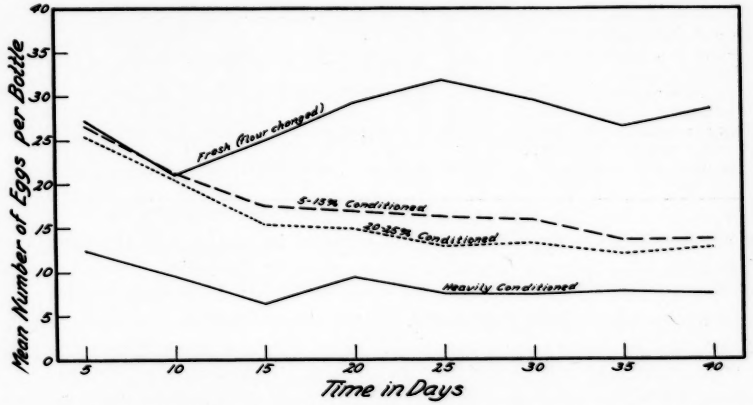

FIG. 1.—Egg production of *Tribolium confusum*, over a 40-day period, living in fresh, 5–15 per cent conditioned, 20–25 per cent conditioned, and heavily conditioned flours.

made to treat each experimental series identically, and all beetles were gently removed from the culture medium before sifting to reduce the possibility of adverse handling effects.

The results of the first experiment are tabulated in Table 1, where the mean rate of egg production (i.e., the mean number of eggs per bottle) per 5-day period is summarized for all eight experimental conditions. The data are also graphed in Figure 1. The beetles in all the environments except the heavily conditioned flour were producing eggs at

about the same rate during the first 5 days of the experiment. There is no marked consistent evidence of environmental conditioning being effective at this time, and it is obvious that the variations between the different bottles and the series are high. It is equally clear, however, that the *Tribolium* in the heavily conditioned medium have had their fecundity significantly lowered during these first few days since they are laying eggs at a much lower rate than the beetles in any of the other groups. In other words, heavy conditioning affects the beetles sooner than does light conditioning. It is a rather curious fact that the fresh conditioned beetles show such a lower rate at the 5-day period than do the fresh series. This difference, although 3.3 times its probable error, is probably due to chance since there is no known differential between these two series at the time of the first egg count. Furthermore, from the performance of the fresh conditioned forms later in the experiment it seems patent that nothing out of the way is behind this possible discrepancy.

TABLE 2

BIOMETRIC CONSTANTS FOR EGG PRODUCTION FOR ENTIRE 40-DAY PERIOD

Flour	Total Number of Eggs Produced for 40-Day Period	Mean Number of Eggs per Bottle per 5-Day Period	σ (Eggs)	C.V. (Per Cent)	n
Fresh (renewed)	9,836	27.3±0.35	10.1±0.24	36.9±0.92	360
Fresh conditioned	3,845	19.2±0.48	10.1±0.33	52.6±1.77	200
Heavily conditioned	1,688	8.4±0.31	6.7±0.21	79.7±2.68	200
5 per cent conditioned	6,594	18.3±0.31	8.8±0.21	48.0±1.20	360
10 per cent conditioned	6,455	17.9±0.29	8.3±0.20	46.3±1.16	360
15 per cent conditioned	6,530	18.1±0.30	8.5±0.21	46.9±1.18	360
20 per cent conditioned	5,910	16.4±0.28	7.9±0.19	48.1±1.20	360
25 per cent conditioned	5,674	15.7±0.26	7.5±0.18	47.7±1.19	360

The 10-day group of egg counts add nothing of importance to the interpretation: there has been a lowering of fecundity among most of the series, but no ordered consistency has appeared. The difference between the beetles in heavily conditioned flour and the other groups is still exaggerated. At the time of the 15-day observations, however, more definite trends are discernible. The fresh-flour populations are decidedly higher with regards to egg production than the other groups; the lightly conditioned environments have become less variable with the 25 per cent culture and less productive than the others; and the beetles in heavily conditioned flour continue to have the lowest rate of egg production.

From the twentieth day to the fortieth day the fecundity becomes more stabilized for all the groups; this can be seen quite clearly from an examination of Figure 1. Apparently, the conditioning effects dealt with here bring about their maximum reduction in fecundity in about 20 days. Thereafter no further marked alterations are noticeable, and the curves maintain a rather constant level during the four remaining egg counts. This is essentially similar to the results described in an earlier paper (Park, 1936), where the influence of 25, 50, and 75 per cent conditioned flour upon fecundity was dealt with. There is also some evidence of differential conditioning between certain of the environ-

ments, with a tendency for the 5, 10, and 15 per cent curves to be higher than the 20 and 25 per cent curves.

This differential effect of conditioning can best be analyzed by grouping all the fecundity rates for each of the environmental series for the entire 40-day period. From these grouped data certain statistical constants are obtainable which aid in describing fecundity as it is influenced by conditioning. Such figures are available in Table 2, where the total number of eggs produced per series, the mean number per bottle, the standard deviation of the distribution, the coefficient of variability, and the number of cases involved in the calculations are recorded. Table 3 makes certain pertinent statistical comparisons between the various mean rates of egg production given in Table 2. These comparisons have been made by calculating the ratio of the difference between

TABLE 3

STATISTICAL COMPARISONS OF THE DATA OF TABLE 2

Comparison	Difference / P.E. Difference	Largest Member of Compared Pair
Fresh with fresh conditioned	13.7	Fresh
Fresh conditioned with 5 per cent	1.6	F-C
Fresh conditioned with 10 per cent	2.3	F-C
Fresh conditioned with 15 per cent	1.9	F-C
Fresh conditioned with 20 per cent	5.1	F-C
Fresh conditioned with 25 per cent	6.6	F-C
Conditioned with 25 per cent	19.2	25 per cent
5 with 10 per cent	0.9	5
5 with 15 per cent	0.4	5
5 with 20 per cent	4.7	5
5 with 25 per cent	6.8	5
10 with 15 per cent	0.4	15
10 with 20 per cent	3.9	10
10 with 25 per cent	5.9	10
15 with 20 per cent	4.2	15
15 with 25 per cent	6.3	15
20 with 25 per cent	1.9	20

any two means to the probable error of that difference, and aid, of course, in evaluating the reliability of such differences.

From Table 2 a number of clear-cut deductions can be drawn as well as certain suggestive points which are not so apparent. In the first place, it is obvious that the fecundity of the *Tribolium* in the two control series—i.e., the high rate of reproduction of the fresh-flour forms and the low rate of the conditioned-flour forms—is markedly different from that of the partial conditioned groups. This is in line with numerous previous experiments (Park, 1934, 1935, 1936) and need not be emphasized here. Also, it is apparent that the fecundity of the beetles in the lightly conditioned and fresh conditioned mediums occupies a median position between the two controls. There is evidence of differential conditioning in that the beetles in the 20 and 25 per cent flours are producing statistically fewer eggs than those in the other lightly conditioned environments. The 5, 10, and 15 per cent series exhibit no significant differentials between themselves with respect to fecundity, i.e., a *Tribolium* in these very lightly conditioned flours pro-

duces about as many eggs in a 15 per cent culture as it does in a 5 per cent culture. There are two possible explanations involved here: either the conditioned flour is not distributed through the fresh in a homogeneous-enough fashion to provide a true differential between the mediums of these three series, or else the flour concentration differences actually do not affect the beetles' oviposition within this range. It seems likely, on the basis of the evidence in general, that the latter explanation is the true one since significant differences are demonstrable between the 5, 10, and 15 per cent groups when contrasted with the 20 and 25 per cent groups, and since previous investigations have shown significant differences between beetles in 50 and 75 per cent conditioned mediums. Table 2 shows no marked differences between the 20 and the 25 per cent environments, and the same explanations apply here as for the more lightly conditioned series.

The oviposition of the beetles in the fresh conditioned populations, where the bottles were initially started with fresh flour without subsequent flour replacement at 5-day intervals, is very interesting. This series was started to see if the beetles would condition their own environment enough to alter their fecundity, as compared with the fresh-flour control, during the 40-day period. Any effect noticeable here would obviously reflect a very light environmental conditioning since the *Tribolium* populations for each bottle are small and short-lived. It is therefore suggestive to note that the beetles of the fresh conditioned series produced significantly fewer eggs than did those of the fresh series but had a fecundity comparable to that of the forms in 5, 10, and 15 per cent flour. This means that very slight conditioning reduces the egg production of *T. confusum*. It further suggests that the alteration of the flour brought about by the beetles in the fresh conditioned bottles is roughly comparable, as regards fecundity effects, to that obtaining in 5, 10, and 15 per cent conditioned flours.

The statistical constants of Table 2 admit of certain interpretations regarding the variation in fecundity displayed by the various experimental series. The coefficients of variability suggest that the relative variation in egg production is significantly lowest for the beetles living in periodically renewed fresh flour; significantly highest for the beetles living in the heavily conditioned flour; and essentially similar for the *Tribolium* living in all the lightly conditioned mediums including the fresh conditioned forms. These facts agree with previous investigations (Park, 1936). Apparently, along with inhibition of oviposition, conditioning of the environment so affects the beetles that they lay eggs more erratically than do forms living in fresh flour. It should be kept in mind that the coefficients of variability may also reflect fecundity variations due to imperfect mixing of the conditioned and fresh flours. In other words, the homogeneity (or heterogeneity) of the medium may influence oviposition variation as well as the flour concentration differences between series. This point has been emphasized in connection with fecundity rates and need not be stressed here.

COMPARISON WITH PREVIOUS RESULTS

Previous experiments (Park, 1936) had analyzed, as already stated, the effects of 25, 50, 75, and 100 per cent conditioned flour upon the fecundity of *T. confusum* and had suggested the present investigation using more lightly conditioned cultures. Since in both of these investigations the experimental results were standardized against the same controls—viz., fresh flour and heavily conditioned flour—and since both were run identically in respect of population size and beetle density, it has seemed interesting to compare the two groups relative to fecundity. Such a comparison based on both

series would give a picture of differential conditioning ranging from very lightly to very heavily conditioned flours. However, when the two series were contrasted on the basis of absolute numbers of eggs produced, it was apparent that the beetles used in the earlier work had a generally higher rate of oviposition than did the forms used in the present report. In other words, it was impossible to make a direct reliable comparison of the two sets of data on the basis of absolute fecundity since the *Tribolium* used in the first experiments displayed an innately higher rate of oviposition than did the later group. This is not an unexpected or unusual experience; fecundity frequently seems to undergo certain periodical fluctuations, and individuals of two experiments not run simultaneously may exhibit different oviposition rates in spite of efforts to control the obvious environmental factors.

In the present case it is the egg production of the *Tribolium* in the two investigations relative to each other which is important. In order to get at this point a simple ratio was calculated which expresses the entire fecundity on a relative scale ranging from 0.3 to 1.8 units. In calculating this ratio for the data of the first investigation a mean rate of egg production per bottle based on all the flour series was obtained. This figure was divided into each of the actual mean rates of oviposition for each 5-day count. For example, the mean number of eggs produced by the 50 per cent series at the first 5-day reading was 34.6, and the mean number of eggs for the entire experiment was 25.7. The ratio for this particular point was then obtained by dividing 34.6 by 25.7, which gave 1.34. This same procedure was followed for every egg count of both bodies of data, using, of course, a different mean figure (17.7) as the divisor when calculating the ratios based on the data reported in this paper. These ratios justify themselves by actually fitting the data of both investigations and allowing them to be expressed on a single relative scale. The results are shown in Figure 2. Since, for the two investigations, the fresh, heavily conditioned, and 25 per cent series were duplicated, a mean for each one of these three groups was obtained, and these are the curves graphed in Figure 2. The fact that the ratios brought the egg production of these control groups so close to one another is further evidence for the reliability of these ratios.

The curves of Figure 2 show, by and large, that as the environments get more conditioned the fecundity of the *Tribolium* is lowered. This holds not only for the heavily conditioned flours but for the lightly conditioned ones as well, and both fall into a moderately well-graded series beginning with fresh flour and ending with heavily conditioned flour. In other words, within the limits of these data, it is possible voluntarily to condition environments and have that factor express itself through a physiological process—fecundity. This expression is not at all precise or closely proportionate to the degree of conditioning, but, considering the nature of the variables dealt with, it does behave consistently enough to demonstrate the importance of differential conditioning in *Tribolium* populations. The most striking contrast is that between the fresh series and any of the conditioned series. It appears that even a slight contamination of the medium with conditioned flour is a potent-enough influence to lower egg production by about one-third. This is a fact of considerable population significance, as will be pointed out later.

REVERSAL OF CONDITIONING EFFECTS

In an earlier paper (Park, 1934) it was shown that *Tribolium* which had been living in conditioned flour and which exhibited low fecundity rates could gradually be returned to normal or control fecundity by replacing them into a fresh-flour environment. Here

the conditioning effect was reversible. This same point has been studied for all series of the present investigation. After the last, or 40-day, egg count had been made, each of the conditioned series (i.e., the fresh-conditioned, 5, 10, 15, 20, and 25 per cent) was split up so that 13 bottles of the 25 received fresh flour while in the other 12 bottles the flour was not renewed. The beetles were not changed in any way and remained in their respective bottles for the entire experiment. Twenty-five (of the original 45) fresh-flour bottles were continued on as before to serve as a control. As previously, egg counts were made at 5-day intervals until four counts, or 20 days, had elapsed. After each count

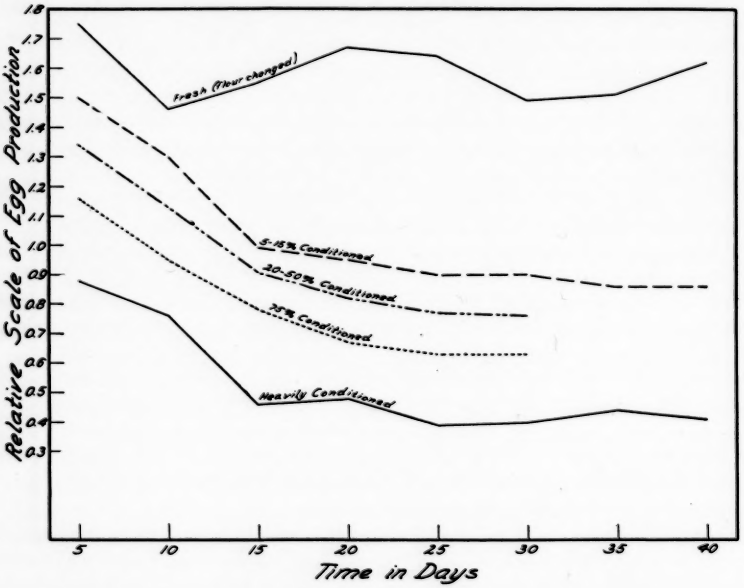

FIG. 2.—Relative egg production (see text) of *Tribolium confusum*, over a 30- and 40-day period, living in fresh, 5–15 per cent, 20–50 per cent, 75 per cent, and heavily conditioned flours.

the flour in bottles 1–13 of all the conditioned series was replenished with 32 gm. of fresh flour without altering the flour in bottles 14–25. This procedure sets up an experiment in which the only known variable is the type of flour, since the past history of the beetles for any one series is identical.

The results of this experiment are presented in Table 4, where the mean rate of egg production per bottle for each of the four counts is summarized; in Table 5, where the biometric constants for the entire 20-day period are tabulated; in Table 6, which lists certain statistical comparisons of the data of Table 5; and in Figure 3, which graphs certain of the mean rates given in Table 4. The latter graph and the tables bring out the essential points rather clearly. In the first place, the decided contrast between the higher fecundity of all the *Tribolium* in fresh flour and that of the forms remaining in their respective conditioned flour is obvious. Those beetles which had been living in

some type of conditioned flour during the first 40 days of the experiment displayed an immediate stimulation of oviposition when returned to fresh flour. This was true for all the conditioned series. It is interesting to note that the forms with a lightly conditioned background reached the control level of fecundity more quickly than did the others.

TABLE 4

MEAN EGG PRODUCTION PER BOTTLE PER 5-DAY READING FOR THE 40–60-DAY PERIOD OF THE EXPERIMENT

Flour	Beetles Returned to Fresh Flour				Beetles Not Returned to Fresh Flour			
	Days				Days			
	45	50	55	60	45	50	55	60
All fresh	33.8±1.64	28.6±1.17	28.7±1.25	30.6±1.36	.....	.....	.....	.....
Fresh conditioned	29.8±2.02	28.1±2.02	31.1±2.11	34.9±2.41	18.6±1.26	18.7±1.07	18.3±1.12	17.7±1.47
5 per cent conditioned	30.7±1.74	28.8±2.05	27.9±2.22	36.5±2.54	18.7±1.61	20.6±1.63	19.2±1.42	21.5±1.79
10 per cent conditioned	26.7±0.84	29.7±0.95	22.7±0.93	25.4±1.36	18.8±0.99	17.3±0.85	17.7±0.87	17.4±0.79
15 per cent conditioned	24.7±1.34	27.2±1.17	22.5±1.17	27.6±1.01	18.7±0.81	16.0±1.34	16.3±0.83	15.3±1.34
20 per cent conditioned	26.6±1.40	27.0±1.70	29.8±1.66	30.0±1.88	17.1±1.30	15.0±0.87	14.9±0.62	17.1±0.93
25 per cent conditioned	24.8±0.99	24.5±1.55	25.3±0.54	27.2±1.06	12.8±1.28	13.7±1.00	13.4±0.93	14.8±1.22
All conditioned	18.3±1.23	22.4±1.38	28.5±1.70	38.2±2.00	9.3±0.99	7.3±0.87	7.5±1.16	9.0±1.20

TABLE 5

BIOMETRIC CONSTANTS FOR EGG PRODUCTION FOR THE 40–60-DAY PERIOD OF THE EXPERIMENT

Flour	Beetles Returned to Fresh Flour					Beetles Not Returned to Fresh Flour				
	Total Number of Eggs Produced for 20-Day Period	Mean Number of Eggs per Bottle per 5-Day Period	Standard Deviation (Eggs)	C.V. (Per Cent)	n	Total Number of Eggs Produced for 20-Day Period	Mean Number of Eggs per Bottle per 5-Day Period	Standard Deviation (Eggs)	C.V. (Per Cent)	n
All fresh	3,046	30.4±0.70	10.4±0.49	34.2±1.63	100	.....	.....	.....	.....	.....
Fresh conditioned	1,611	30.9±1.13	12.0±0.79	38.8±2.59	52	879	18.3±0.63	6.5±0.44	35.5±2.46	48
5 per cent conditioned	1,610	30.9±1.15	12.2±0.81	39.4±2.63	52	959	19.9±0.82	8.5±0.57	42.8±2.97	48
10 per cent conditioned	1,358	26.1±0.58	6.2±0.41	23.7±1.57	52	854	17.7±0.48	5.0±0.33	28.2±1.95	48
15 per cent conditioned	1,326	25.5±0.63	6.7±0.44	26.2±1.74	52	796	16.5±0.59	6.1±0.41	36.9±2.56	48
20 per cent conditioned	1,474	28.3±0.86	9.2±0.60	32.5±2.16	52	769	16.0±0.54	5.6±0.38	35.0±2.42	48
25 per cent conditioned	1,324	25.4±0.58	6.2±0.41	24.4±1.62	52	656	13.6±0.58	6.0±0.41	44.1±3.06	48
All conditioned	1,396	26.8±1.08	11.5±0.76	42.9±2.85	52	397	8.2±0.55	5.7±0.38	69.5±4.82	48

The fresh conditioned and 5 per cent series were producing eggs at a control rate at the time of the first (i.e., 45-day) reading. The 10, 15, 20, and 25 per cent groups had reached this level at the second and third readings, while the *Tribolium* reared in heavily conditioned flour took longer to increase their fecundity. Once there, however, the latter beetles exhibited just as high a rate of oviposition as any of the other series; their mean rate of egg production for the 20-day period did not differ significantly from that of the control group. In other words, the inhibition of fecundity is reversible, and the time required to restore the control rates varies with the rigorousness of past conditioning. The

statistical comparisons of Table 6 show that the differences in fecundity between the beetles in fresh flour and those in some type of conditioned flour are highly significant.

TABLE 6

STATISTICAL COMPARISONS OF THE DATA OF TABLE 5

Comparison	Difference / P.E. Difference	Largest Member of Compared Pair
Fresh with all conditioned (renewed)	2.8	Fresh
Fresh conditioned with fresh conditioned (renewed)	9.8	Renewed
5 with 5 per cent (renewed)	7.8	Renewed
10 with 10 per cent (renewed)	11.3	Renewed
15 with 15 per cent (renewed)	10.5	Renewed
20 with 20 per cent (renewed)	12.1	Renewed
25 with 25 per cent (renewed)	14.5	Renewed
All conditioned with all conditioned (renewed)	15.5	Renewed

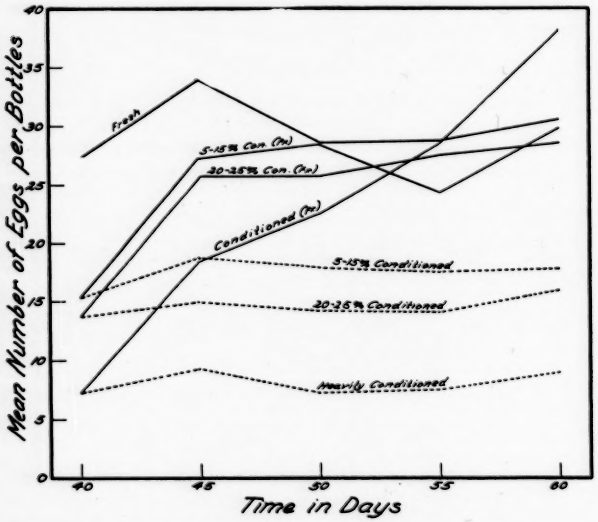

FIG. 3.—Egg production of *Tribolium confusum*, over a 20-day period, of (*a*) forms living in fresh flour which had spent 40 days in some type of conditioned flour (except the fresh series) and (*b*) forms living in 5–15 per cent, 20–25 per cent, and heavily conditioned flours (*a*, in solid lines; *b*, in dotted lines).

This substantiates the conclusion presented earlier that even a slight amount of environmental conditioning is detrimental to fecundity.

The fecundity curves for the beetles remaining in conditioned flour, in addition to furnishing a control for the foregoing group, also make an instructive adjunct to the 40-day experiment. These results can be looked upon as a continuance of the original

experiment, so that a 60-day total period instead of a 40-day one may be discussed. The chief fact added by the last four readings is that the fecundity rates earlier established do not change greatly for any of the series, i.e., the apparent equilibrium reached by the end of the first 20 or 25 days is continued through the 60-day period. A comparison by series of the mean egg-production rates of Table 2 with those of Table 5 shows very little distinction between the oviposition during the 40-day period and that during the last 20-day period. The only possibly significant differences occur within the fresh and 25 per cent series, where the deviations are 3.9 and 3.4 times their probable errors, respectively. These differences, however, are not large enough to affect the general trend of the fecundity curves. If a series of mean rates of egg production based on the entire 60-day period are obtained, they fail to alter materially the general conclusions, with regard to differential flour conditioning, drawn earlier from the 40-day material. These 60-day means with their probable errors can be listed as follows:

Fresh	28.0±0.24	15 per cent conditioned	17.9±0.31
Fresh conditioned	19.0±0.43	20 per cent conditioned	16.3±0.26
5 per cent conditioned	18.5±0.24	25 per cent conditioned	15.5±0.29
10 per cent conditioned	17.9±0.27	Heavily conditioned	8.4±0.27

A group of statistical comparisons similar to those of Table 3 based on the foregoing data suggest no problems not already discussed.

The coefficients of variability of Table 5 substantiate the conclusions already reached about the effect of conditioning on egg-laying variation. In general, the C.V.'s are higher for the beetles continued in conditioned flour than they are for the forms introduced into fresh flour. Apparently, just as the fresh flour increases the fecundity of the *Tribolium*, it also decreases the variability of their egg production.

EGG FERTILITY AND CONDITIONING

From the viewpoint of population trends it is as important to have information about the fertility of eggs (i.e., the number of eggs hatching into larvae) as it is about the fecundity or total number of eggs produced. In earlier investigations this point has been studied by segregating the eggs after counting and placing them to one side until hatching. Knowledge of the total number of eggs segregated and the total number of larvae emerging from those eggs allows the calculation of a fertility percentage. The generalization growing out of these previous data has been that fertility is not greatly or consistently affected by conditioned flour. Even though *Tribolium* inhabiting highly conditioned mediums have their oviposition greatly lowered, their eggs, once laid, have just about as good a chance of hatching as have the eggs laid by fresh-flour beetles. This is similar to the experience reported by Pearl (1932) for *Drosophila*, who found that it was the fecundity of the flies and not their fertility which was primarily affected by population density.

Fertility data also have been collected for the present experiments by isolating eggs after counting into fresh flour. The eggs were not isolated by bottles but by series and by counts, e.g., all the eggs produced by all the beetles in the 5 per cent conditioned flour at the time of the first 5-day count were grouped together into one container. The same procedure was followed for each of the other series at the 5-day count and repeated for all of them on each successive period up through the 40 days. No fertility readings were made after 40 days. After allowing adequate time for the fertile eggs to hatch, the flour

was sifted and the larvae spread on cross-hatched bristol board and counted. A condensed summary of the results appears in Table 7, where the total number of eggs produced during the 40 days by the eight series, the total number of larvae, and the fertility percentages are recorded. The mean egg fertility for the present data is slightly higher than that obtained in previous studies. Park recorded mean fertility percentages of 71.4 and 65.6 per cent (1935) and 70.3 per cent (1936) as compared with the 76.3 per cent of Table 7. These rates, however, are close enough to be reasonably certain that no excessive differential exists between the various experiments with respect to fertility. As before, it is not possible to point out any consistent relation between conditioned flour and fertility. While there are certain deviations between the fertility percentages for the various series, and while these deviations differ significantly from an expected value as compared by the χ^2 test, it does not seem possible to draw any conclusion about fertility

TABLE 7

EGG-FERTILITY DATA BASED ON THE ENTIRE 40-DAY EXPERIMENT

Series	Total Number of Eggs Produced during the 40 Days	Total Number of Larvae Hatched during the 40 Days	$\frac{\text{Larvae}}{\text{Eggs}} \times 100$ (Per Cent)
Fresh	9,836	7,618	74.4
Fresh conditioned	3,845	2,962	77.0
5 per cent conditioned	6,594	4,997	75.7
10 per cent conditioned	6,455	4,957	75.1
15 per cent conditioned	6,530	5,108	78.2
20 per cent conditioned	5,910	4,666	78.9
25 per cent conditioned	5,674	4,609	81.2
All conditioned	1,688	1,184	70.1
Mean	.....	.....	76.3

other than that it is not greatly affected by conditioning. This conclusion must be tempered, for the time being, by the fact that in each fertility experiment performed to date the percentage of eggs hatching into larvae was slightly lower for the heavily conditioned series than for the fresh series. It is possible that this is a point of some significance although at the moment the data suggest otherwise.

DISCUSSION AND SUMMARY

Since a good deal of experimental work dealing with the effect of conditioned flour on certain physiological processes of *T. confusum* has appeared in this and earlier papers (Park, 1934, 1935, 1936, 1936*a*), it seems advisable at this point first to summarize briefly this material and second to relate it where possible to population dynamics. The principal facts which have grown out of these investigations are arranged in the following protocol:

I. Flour taken from run-down *Tribolium* populations (i.e., heavily conditioned flour) has the following known relations to the beetles' physiology:
   A. It reduces their cannibalism (i.e., the rate at which the imaginal beetles eat their own eggs) to about half that of control beetles living in fresh flour.

B. It reduces their fecundity drastically—lowering it three or more times below a control level; quickly—some effect can be obtained within 5 days' time and the maximum effect within 20 days; and reversibly—the egg production can be returned to a control level by reintroduction of the beetles into fresh or unconditioned flour. It also reduces the fecundity of virgin female beetles, i.e., forms which do produce some eggs but which have never copulated.

C. There is some evidence that conditioning affects fecundity through the males as well as through the females since in one group of experiments the data suggested that fresh females, mated to conditioned males, had a lower rate of reproduction than did fresh females mated to fresh males.

D. It increases the relative variability of their egg production.

E. It apparently does not alter in any consistent fashion the fertility of the eggs. That is, once produced, eggs from fresh-flour beetles have about as good a chance of hatching as eggs from conditioned-flour beetles and vice versa.

F. It increases the duration, variability, and mortality of larval metamorphosis.

G. It does not significantly alter the oxygen consumption of imago *Tribolium* since there is no apparent difference between the respiratory rate of beetles reared in fresh flour as compared with those reared in conditioned flour. A definite sexual difference, however, was demonstrable with the females using more oxygen than the males.

II. "Dilution" or grading of this heavily conditioned flour to form a series of differentially conditioned environments ranging from fresh conditioned (original fresh flour not renewed during the course of the experiment) through 5, 10, 15, 20, 25, 50–75 per cent conditioned and compared against fresh and heavily conditioned flour controls brings out the following points about the productivity of *T. confusum:*

A. The fecundity of the beetles is roughly proportionate to the concentration of the conditioned flour in the medium. In other words, as the flour gets more conditioned the rate of oviposition lowers.

B. This is not a uniformly proportional relationship. There are certain regions where the change in the percentage of conditioned flour produces no significant effect on fecundity and others where the effect is marked. For example, the beetles in the 5, 10, and 15 per cent conditioned environments produce eggs at the same rate, yet these three groups are far below the forms in fresh-flour and significantly above the forms in the more conditioned series.

C. The fecundity of the *Tribolium* living in the fresh conditioned, 5, 10, 15, 20, and 25 per cent mediums also can be raised to that of the fresh-flour control by placing the beetles in fresh flour. It is instructive to note that the forms in the most lightly conditioned environments reach the control level sooner than do the beetles in heavily conditioned flour but that the latter eventually come to oviposit at as high a rate as the former.

D. No consistent effect of differential conditioning on egg fertility is demonstrable.

The foregoing outline summarizes the various known physiological effects of conditioned flour on *T. confusum* but makes no attempt to evaluate them from the viewpoint of population dynamics. Such an evaluation is the second task of this discussion. The original emphasis behind the study of the conditioned-flour problem grew out of the following facts: first, that *Tribolium* populations decline as they get older if the flour is not periodically renewed, and, second, that the flour in which these populations are living becomes more conditioned with age. The latter point was recognized by Chittenden in 1896, who, in commenting on the biology of *Tribolium*, said, ". . . . When the insects have time to propagate, they soon convert the flour into a gray, useless mass." It logically follows from these two premises that conditioning is an expression of population decline—a density expression, as it were—since it is the number of beetles them-

selves which is responsible for the rate of conditioning of the flour. As conditioning does go hand in hand with population decline, the question is raised: Is it a result, a cause, or both? That it is a result of decline seems patent because, as already mentioned, it is an expression of population age and density. That it is a cause of decline could not be answered on a priori grounds since no knowledge of the physiological effects of conditioning on the beetles themselves could be deduced without experimentation. Such experimentation has unearthed the material summarized in the foregoing protocol. The immediate task is to sort these facts and point out which ones, if any, are important in explaining the decline of *Tribolium* populations and which are merely physiological effects not directly related to population trends.

It appears obvious that environmental conditioning is not influencing population decline through the medium of cannibalism (egg-eating). Even though there is a definite effect on this process—i.e., the beetles consume fewer eggs if they are living in conditioned flour than they do in fresh flour—it selects against population decline since more eggs escape being eaten and thereby have a better chance of hatching into larvae. Likewise, the relation of conditioning to egg fertility is not important from the population viewpoint since it has been impossible to demonstrate experimentally that the viability of the eggs is consistently greater in one type of medium than in another. The metabolic studies on oxygen consumption also suggest nothing of value in explaining population decline.

It has been pointed out by several population students, notably Pearl (1927), that fecundity is one of the two primary biological variables involved in population dynamics (the other variable being mortality), and that any factor influencing this process must be regarded as essential. On the basis of the experimental knowledge available it seems that the effects of conditioning on fecundity, as well as on larval metamorphosis, are highly important. It is not the purpose of the authors to suggest that these are the only factors involved or that conditioned flour is the sole cause of population decline. The data are too limited to support such a view, and other collateral possibilities suggest themselves. It does seem inescapable, however, that the drastic inhibition of fecundity and the increase in larval mortality induced by conditioned flour are real and important forces contributing to the senescence of *Tribolium* populations. The experiments with differentially conditioned flour have added the points: first, that the effect of conditioning on fecundity is cumulative, i.e., it becomes more drastic as the flour gets more conditioned, and, second, that a slight amount of conditioning is detrimental to the oviposition rate. The latter point suggests that *Tribolium* cultures are rarely in an optimal situation with respect to their fecundity since the flour usually is at least partially conditioned. It is possible, however, that other factors (e.g., the large numbers of eggs relative to the few adult beetles and the subsequent reduction of opportunities for cannibalism) compensate for this during the earlier stages of population growth and so keep conditioning, working through fecundity, from being an important influence until later. It is also possible, although there exist no data for it at present, that conditioned flour in certain optimal concentrations may have a stimulating effect upon the *Tribolium* which would be important in their population dynamics. This type of result is yielding more and more to investigation, as has been ably pointed out by Allee (1931, 1934).

It is realized that there are certain justifiable criticisms of the conditioned flour investigations, and it is not the purpose of the authors to gloss over this discussion without mentioning some of them. In the first place, no accurate analysis or synthesis of condi-

tioned flour is available at the moment; the emphasis to date has been placed on making biological assays of conditioning and not on physical-chemical studies. This means that it is not possible to attribute the effects of conditioned flour to anything more specific than the flour itself. Second, there are certain objections to "diluting" heavily conditioned flour with fresh flour and assuming that the resultant medium closely simulates a type of environment produced by the actual beetle culture. The points in favor of this procedure are, first, that the actual results obtained do suggest that a real environmental gradation has been set up, and, second, that in making "dilutions" from two standards (the fresh and heavily conditioned flours) the composition of the intermediate concentrations, relative to these standards, is rather accurately known. It is hoped that future investigations, both analyses of the conditioned flour itself and studies on other physiological processes of population significance (e.g., mortality), will further this program and remove some of these objections.

The authors wish to express their sincere appreciation to Professor Raymond Pearl for reading the manuscript.

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CYTOLOGICAL STUDIES OF THE DECIDUAL REACTION IN THE RAT DURING EARLY PREGNANCY AND IN THE PRODUCTION OF DECIDUOMATA[1]

(Sixteen figures)

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IMPLANTATION

THE greater part of our knowledge concerning the decidual cells formed in the antimesometrial wall of rodent uteri during early pregnancy has been obtained from observations of that organ in its relation to the attachment and growth of the embryo. Since Tafani (1886) and Duval (1891) noted this reaction on the part of the mouse and rat uterus, numerous investigations have been added to the published material dealing with the subject. However, very few of these publications deal with the uterine mucous membrane except in so far as it aids in the explanation of the embryonic activity. The histological value of the work of Spee (1901) on the guinea pig and Burckhard (1901) on the mouse is well known. Jenkinson (1902), concerned chiefly with the fetal giant cells, gave a sketchy account of the glycogen and lipoid content of the mouse uterus. From these and other studies we know that in many rodents an antimesometrial decidua develops.

L. Loeb (1907) discovered that the antimesometrial wall of the guinea-pig uterus produces decidual tissue after mechanical injury. This has been abundantly verified. Although he recognized that the experimentally produced enlargements resembled decidua, he regarded them as tumors and called them "deciduomata." Unfortunately, the term, with all its implications, has been established by general usage.

Lataste (1887), in his observations of the breeding habits of rodents, noted that they would mate following parturition and that: "Ches les Muridés, quand la femelle entre en lactation au début de sa grossesse, la durée de la gestation est de trois périodes génitales; dans tous les autres cas, elle n'est que deux périodes." His *périodes génitales* were 10–11 days long. In explanation of this he postulated: "Les ovules après fécondation, ont leur développement arrête pendant la première des trois périodes génitales de cette gestation." Duval (1891) provided evidence of the same in the rat. Kirkham (1916) and Enzmann, Saphir, and Pincus (1932) have confirmed Lataste's postulation by showing that the delay is due to the failure of the embryo to become attached at the proper time —a phenomenon which when explained, I believe, will certainly find many if not all its basic factors in the state or condition in which the uterine mucous membrane is at that time.

The explanation of the various interpretations may be found in the lack of an ade-

[1] I wish to thank Dr. G. W. Bartelmez for his constructive criticism during the course of the work and for his continued encouragement. The drawings are the work of Miss Agnes Dixon.

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quate anatomical knowledge of this tissue. Consequently, the problem has been approached from a cytological viewpoint in an endeavor to provide in part an anatomical foundation which may be used in the correlation of the various factors involved.

TABLE 1

Rat No.	Gestation Age		Rat No.	Gestation Age	
	Day	Hour		Day	Hour
1	7	23	46	7	22
2	7	12	47	11	1
3	6	15	49*	9	12
4	6	15	50	10	23
5	6	18	51†	6	11
7	7	23	52†	7	12
8	7	.....	53†	11	.....
11	8	21	54*	9	.....
12	6	.....	57†	8	20
15	7	.....	58†	8	12
16	7	18	61†	6	6
19	7	12	62†	6	2
20	6	23	63†	6	8
25	10	12	64†	6	11
26	5	12	65†	6	2
27	9	11	67†	7	23
28	7	22	68†	6	15
29	8	20	69†	7	13
30	7	8	70†	7	.....
31	10	12	72†	6	18
33	10	.....	73†	6	21
35	8	12	77†	8	12
36	7	3	79*	9	22
37	6	2	80*	8	13
38	6	6	81†	12	1
39	9	18	82†	6	19
40	8	5	83†	10	15
41	12	.....	85†	10	12
44	6	12	86†	6	14
45	6	4			

* Denotes animals in which deciduomata were produced in 1 (pregnant) horn.

† Denotes animals in which 1 horn was kept empty for deciduoma studies by tying off and removing part of the Fallopian tube.

In bilateral pregnancies the number of embryos varies from 3 to 13 with an average of 7.2 embryos per litter.

MATERIAL AND METHODS

Gestational ages were dated from the time of ejaculation. The use of this method introduces no significant error, since the variations noted between embryos of the same copulation age, obtained from different animals, were seldom greater than those found in a single horn. Table 1 gives the number of animals used and the gestation age of each.

The animals were usually killed by a blow over the head, the uterus removed, and the loci of implantation fixed in various fluids while the animal was still alive or very few minutes after death. A few vascular profusions of the fixative were done. In order to

determine the vascular pattern of the decidua, an 8 per cent solution of colloidal mercuric sulphide was injected into the internal jugular vein while the animal was under ether, the uterine blood vessels were clamped off, and the organ removed to the fixing fluid (Daron, 1936).

Numerous fixations were used with varying degrees of success. The most valuable proved to be Bensley's modification of Zenker (2.5 per cent bichromate, 5 per cent mercuric chloride, and 10 per cent formol) followed by a 3–5 per cent potassium bichromate mordant. This fixative not only preserved the general histological structures but also proved most adequate in the preservation of lipoins and mitochondria. Regaud and a 10 per cent formalin solution were also used in lipoid preparations by the Sudan III method. The former is good, whereas formalin introduces difficulties in sectioning the material. Müller's fluid and Maximow's formol-osmic Zenker were used in osmic methods for lipoid.

Romeis' (1929) solution of 40 per cent Sudan III, followed by Erlich's haematoxylin, was used in staining lipoins in frozen sections. With a few alterations of Zwemer's (1933) method, sections from 3 to 8 μ in thickness were obtained. The sections were removed from the knife and placed upon a clean slide. Sufficient water to straighten the sections was added, and the whole allowed to dry at room temperature. This leaves a thin film of gelatin over the tissue, which preserves it as well as paraffin and may be soaked off in water without the loss of sections. If too much water is added, the tissue is not covered by gelatin and cracks, whereas if not enough is used, too much gelatin remains, and the sections are likely to fall off. Zwemer's suggestion of using formalin proved disadvantageous because the formalin leaves the gelatin insoluble in water, thus masking the staining of the material. Sections thus obtained may also be stained by other methods, especially for the sharp differential staining of mitochondria.

In addition to the formol Zenker solution, Maximow's fluid, followed by 3–5 days' osmication and subsequent bleaching in potassium permanganate, was also used in the demonstration of mitochondria which were stained with aniline acid fuchsin followed by Mallory's aniline blue stain. Fresh tissues, sometimes stained supravitally with janus-green B for mitochondria, were studied as a control of the fixed material.

One part formol to nine parts absolute alcohol (O'Leary) was used in fixing glycogen which was stained with Best's carmine. Saliva-digested controls were made in each case. In some specimens the iodine reaction was also used. Weaker alcohol solutions failed to preserve all the glycogen. Good staining of chromidial substance with toluidin blue was obtained after absolute alcohol formol fixation. Reticulum fibers were demonstrated by the Bielchowsky-Foote silver method after formol Zenker fixation.

OBSERVATIONS

A review of the histology of implantation is necessary to clarify the nomenclature to be used. A general survey of the process is presented in Figure 1, which diagrammatically shows the antimesometrial and mesometrial decidual formations which differ in character and in time of appearance.

EPITHELIAL CHANGES

During the fifth day, before implantation, the uterine epithelium is uniform throughout. With the start of the sixth day, a series of transformations in the uterine surface epithelium occurs which divides it into four areas: (1) the *antimesometrial area*, which becomes pseudostratified; (2) the *implantation area*, where the epithelium surrounding the embryo flattens to a low cuboidal or squamous type; (3) the *transitional area*—ap-

proximately half of the epithelium lying mesometrial to the embryo shows a transition from the high columnar epithelium of fifth-day cases to the low cuboidal found in the implantation area; and (4) the tall columnar *mesometrial area.*

With this differentiation the diameter of the implantation chamber has become greater than that of the remainder of the lumen. With the continued growth of the embryo the implantation area is further stretched. The first replacement of this epithelium is brought about by the migration of a single trophoblastic cell between the flattened epithelial cells none of which is destroyed at first. This trophoblastic migration continues until at the end of the sixth day the trophoblast is in contact with the stroma throughout the implantation area. At that time the base of the embryo, the ob-placental pole, lies on an antimesometrial epithelial shelf, the sides are in contact with the stroma, and the Träger touches the lowermost cells of the transitional area. It is to be noted that the epithelium forms a blunt wedge between the embryonic and the maternal tissue at the antimesometrial and mesometrial edges of the denuded area.

During the seventh day the antimesometrial area undergoes a fatty degeneration which is completed some time during the eighth day (cf. p. 220). While this is going on, the lumen continues to lengthen along the implantation axis (cf. p. 218), whereas the epithelial cells do not multiply. Thus, more and more surface is to be covered, and the epithelial cells of the transitional area become lower; their nuclei become first spherical and then flattened.

Until the beginning of the eighth day the alterations in the transitional area are merely changes in cellular form. However, at this time there has been some extravasation of blood into the lumen. The blood lifts the lowermost portion of the transitional epithelium from its basement membrane, and fatty degeneration ensues. By the middle of the ninth day it is gone. It is not until late in the tenth day that the epithelium becomes continuous antimesometrially to the implantation site.

The high columnar cells of the mesometrial area rest upon a reticular basement membrane and have definite cell outlines and terminal bars. The oval nuclei are basal. The cytoplasm is abundantly supplied with spheroidal, rodlike, and filamentous mitochondria with a predominance of the latter. In 4μ sections they fill the distal end of the cell, making it difficult to distinguish the individual mitochondria. At the sides and basal to the nucleus they tend to be longer and thicker filaments lying parallel to the long axis of the cell. There is no change in the mitochondria when differentiation in cellular form occurs in the various areas. Normal-appearing mitochondria may be found in cells lying next to the migratory trophoblastic ectoderm. Since these uterine cells are also normal in other respects, there is no evidence of injury during the early phase of invasion. During the seventh day the increasing amount of lipoid in the cells of the antimesometrial area makes it impossible to demonstrate normal mitochondria, if any are present. The same is true of the greater part of the transitional area after the eighth day.

STROMA CHANGES

Just before embryonic attachment occurs, early in the sixth day, the changes in the stroma become localized and orientated about the implantation chamber. Thus, a line drawn through the mesometrium and the center of the embryo passes through the central axis of decidual activity and may be called the "implantation axis." These changes consist of an enlargement of the subepithelial cells into decidual cells, a differentiation of the capillary bed, and a localization of glands which enable one to distinguish an antimesometrial and a mesometrial region (Fig. 1, *A* and *B*).

The enlargement of the subepithelial cells begins late in the fourth day, and by the time the embryo has reached its definitive position (last half of fifth day) the "primary

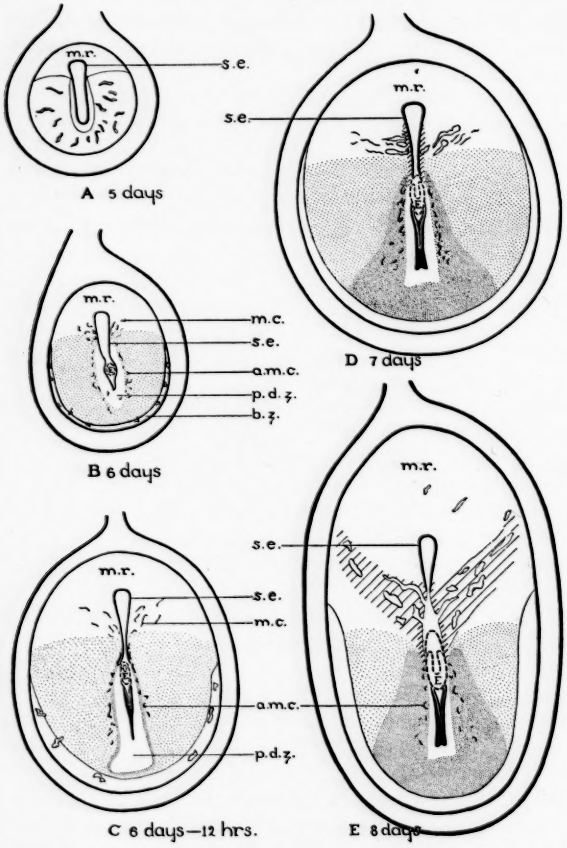

FIG. 1.—A series of diagrammatic cross-sections to show the differentiation and development of the various regions of the decidua: *A*, 5 days; *B*, 6 days; *C*, 6½ days; *D*, 7 days; *E*, 8 days; *F*, 9 days; *G*, 10 days; and *H*, 11 days. It is to be noted that the growth of the antimesometrial (lipoid) region consists of two phases: (1) an early, but brief, development about the implantation axis differentiates the primary decidual and implantation zones; and (2) a second phase of growth differentiates the secondary decidual zone which ultimately forms the decidua capsularis. The development of the mesometrial (glycogenic) region progresses rapidly late in the seventh day and soon becomes the dominating feature, giving rise to the maternal placenta. Embryonic structures (*E*) are represented by broken lines. × 13.

decidual zone" (Fig. 1, *B* and *C*, *pdz*) is differentiated, and only small intercellular spaces and reticulum separate the closely packed cells. The primary decidual zone extends in the implantation axis for varying distances antimesometrially.

The primary decidual zone is comparatively free from prominent capillaries, and as it spreads they gradually recede from the lumen. In early sixth-day cases a narrow mesh-

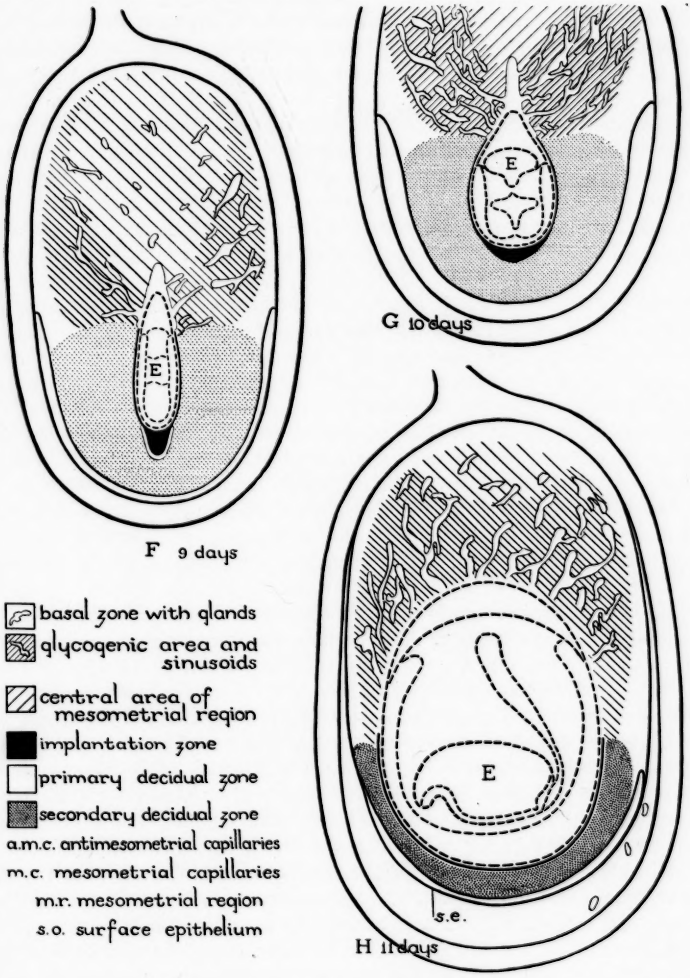

FIG. 1.—*Continued*

work of capillaries surrounding the primary decidual zone becomes pronounced. At the mesometrial extremity of the zone the capillaries extend to the epithelium (Fig. 1, *B*, *amc*), and here their radial arrangement accentuates the differentiation of mesometrial and antimesometrial regions, which are further differentiated by the presence of glands in

the antimesometrium. Between the glands are loosely associated stellate stroma cells and a heavy reticulum, which stains metachromatically with toluidin blue. During the last half of the fifth day and the first few hours of the sixth, the stroma surrounding the primary decidual zone becomes highly oedematous, and the glands are pushed to the sides of the enlarging area. Thus, in cross-sections through an implantation site they are found next to the antimesometrial musculature in a narrow strip of inactive stroma which may be called the "basal zone" (Fig. 1, *B*).

During the first half of the sixth day the development of the antimesometrial region results in a localized thickening of the uterine horn. The rapidly differentiating primary zone spreads to the basal zone. However, the cells at the core of the decidual zone lag in their development and stain more intensely, thus differentiating an additional zone which may be called the "implantation zone." This is indicated in solid black in Figure 1, *C*. The reticulum of this zone is radially organized, while in the larger-celled primary zone its meshes are irregular and wide. However, the fibers in both zones have lost their metachromatic reaction to toluidin blue—a characteristic feature of the decidual zones as they increase in extent. The capillary meshwork at the sides of the primary zone retains its position as the latter enlarges during the sixth day (Fig. 1, *C*, *amc*).

Before the middle of the sixth day a new decidual development appears at the periphery of the primary zone and is characterized by its lipoid-laden decidual cells. This secondary decidual zone at first undergoes an extensive hyperplasia, and many binucleated cells appear. Hypertrophy continues through the last of the seventh and eighth days to reach a climax during the ninth day and clearly differentiates the antimesometrial region (Fig. 1, *F*, stipple). The formation of binucleated cells throughout the secondary decidual zone is a feature, presumably, of the rapid hyperplasia since no indication of amitosis or cell fusion has been observed. Two nuclei, but never any more, may be present in small as well as in large decidual cells of this zone.

The changes occurring in the primary zone are definitely associated with the enlargement of the implantation chamber. As it increases in size, its pressure and that of the growing tissue of the secondary zone cause the primary zone to narrow, as its cells which border the implantation chamber flatten (Fig. 1, *E*). Some cells are degenerating and have pyknotic nuclei and a fibrous cytoplasm. With this narrowing the capillaries reach the surface of the implantation chamber at the beginning of the eighth day and are open so that extravasation into the lumen occurs. In this connection it is to be noted that fetal giant cells lie against maternal tissue and at the open mouths of the capillaries.

The loss of tissue along the sides of the implantation chamber continues but soon becomes secondary to the degenerative process in the implantation zone. This zone, as well as the primary zone, shortens during the rapid growth of the secondary zone. Many of its cells, especially those which have replaced the antimesometrial epithelium during the eighth day, undergo a fatty degeneration, and the others become fibrous. By the middle of the ninth day numerous intact capillaries appear in the implantation and primary zones, some of which are subsequently opened, allowing extravasation.

The capillary meshwork adjoining the primary zone becomes less distinct as the secondary zone grows. By $9\frac{1}{2}$ days the latter is supplied by a uniform and purely capillary net; in thin sections its meshes inclose 2–4 decidual cells from which the large endothelial cells are separated only by an occasional reticulum fiber. Before the ninth day the cells of the secondary zone vary in form and size even within small areas. During this day they become uniform, and the tissue superficially resembles liver.

As the cylindrical implantation chamber becomes spherical during the ninth and tenth days, the implantation zone spreads out over its surface (Fig. 1, *F* and *G*). Cells degenerate here and along the surface of the implantation cavity. However, the rapid increase in the size of the cavity is the greatest factor in the thinning of the antimesometrial decidua. Owing to this pressure the cells become elongated or rectangular in form by the eleventh day, and by the twelfth day the antimesometrial decidua has been reduced to a thin decidua capsularis.

During the last of the seventh day rapid growth begins in the mesometrial region. It is due to an increase in oedema and to cellular hypertrophy. Since the growth and sinusoid formation are associated with the deposition or elaboration of glycogen, it will be considered below (cf. p. 222). The general direction of the growth may be seen in Figure 1 (*E*, *F*, *G*, and *H*) where the area is represented by hatching.

LIPOID

Typical stages of development will be considered in the description of the lipoid. Although both osmic and Sudan III preparations have been made, only the latter will be described. The antimesometrial decidua is particularly distinguished by its early accumulations of lipoid.

Five-day–22-hour stage.—The general appearance of the stroma is shown in Figure 14. Only the high columnar surface epithelial and gland cells show lipoid. Large lipoid spherules are crowded between the nucleus and the base of the cell, and a few lie just distal to the nucleus. The distal third of the cell does not contain any large spherules; however, minute rodlike granules are present and, under low magnifications, give this portion of the cell a yellowish coloration. This is true in the gland cells also in which only a few spherules are found. In a few sections of a somewhat younger specimen the decidual cells of the primary zone, which have not reached their full size, contain from 6 to 24 minute spherical lipoid granules not much larger than mitochondria. Occasionally they are all at one side of the nucleus. The cells of the secondary decidual zone also have a few small granules.

Six-day–4-hour stage.—The surface epithelium has differentiated into its four areas (cf. p. 214). The mesometrial area is the same as in the fifth-day case (Fig. 8). The transitional area shows a decrease of lipoid in the distal portion of the cell in which the basal spherules are somewhat larger (Fig. 7). The pseudostratified epithelium of the antimesometrial area has a high content of large basal spherules (Fig. 6). The flattened cells of the implantation area have relatively as much lipoid (Fig. 9).

The implantation zone is now differentiated by the fact that its cells are comfortably filled with granules two to three times the size of those in the primary decidual zone where they have increased somewhat in number and size as compared with the previous stage. In the secondary zone and in the mesometrial region the majority of the cells have a few minute granules.

Six-day–9-hour stage.—Relatively the implantation zone has not increased in lipoid content. The increase in the primary zone is due to the increase in the size of individual granules which occupy an area just around the nucleus or at one side of it. The cells adjacent to the primary zone and a few others have hypertrophied and contain a few large spherical granules in addition to the minute granules which characterize most of the secondary decidual zone. No change has occurred in the mesometrial zone.

Six-day–15-hour stage.—The implantation epithelium is gone. In most of the cells of

the transitional and mesometrial areas, the basal granules are missing, whereas the distal half of the cell still contains lipoid. In an implantation site of the same age from another animal the mesometrial and transitional epithelia are quite high and contain an abundance of basal spherules. In the lower third of the transitional area the whole cell is packed with spherules. The antimesometrial area is short and shows both pseudostratification and a heavy lipoid content.

The cells of the implantation zone are filled with large lipoid spherules which may lie separated or in groups, and often it seems as if there had been partial fusions. Under a low magnification these cells are easily mistaken for epithelial cells of the antimesometrial area. The primary decidual zone extends a considerable distance beyond the mesometrial end of the embryo (Fig. 1, *D*). Here the cells are elongated in the direction of the implantation axis. The few lipoid granules lying at one end of the nucleus approach the size of the spherules of the epithelium. At the antimesometrial end of the zone the cells have not changed.

Under low powers the lipoid in the secondary decidual zone is easily recognized. In addition to the minute granules previously found there are larger granules which may appear as spheres, crescents, or rings. They tend to be localized at one side of the cell, or they may be evenly dispersed throughout the cytoplasm. Occasionally a few fuse to form an elliptical globule. In dividing cells the granules move to the periphery and are never found in the spindle area. The binucleated cells do not differ from the rest in the content or nature of the lipoid. It is to be remembered that, although the zone is rather uniform, individual cells of any form or size, lying by themselves or in groups of two or three, may be found which are free of lipoid. Again, it is possible to find a small cell heavily loaded with granules while an adjoining cell, twice its size, may have only a few or no granules. These conditions hold true as long as the lipoid remains granular.

If a cross-section perpendicular to the implantation axis were made through the widest point of the lipoid area, the implantation zone would form a central disk heavily loaded with lipoid. Surrounding that would be found a ring poor in lipoid—the primary decidual zone. The secondary zone would form a third ring, again heavily stained with a gradual decrease toward its outer edge. The mesometrial region shows little change in lipoid content.

Seven-day stage.—The mesometrial and transitional epithelia still contain lipoid spherules, but the number is rapidly decreasing, and most of the cells of the mesometrial area show only the minute granules. Antimesometrially only a few free epithelial nuclei are found among the large, closely packed lipoid spherules. This region is quite distinct from that in which the embryonic giant cells lie. They, too, contain large lipoid spherules.

The primary decidual zone differs but little from that of the previous case. The elongate cells of the implantation zone nearest the implantation cavity take a yellowish stain, and only a few orange-colored granules are present. Here and there a cell lying free in the cavity is loaded with lipoid spherules. It is impossible to distinguish epithelial from decidual cells at this point. The remainder of the implantation zone, which is relatively narrower and shorter than in the previous case, shows no change in its lipoid content.

Practically all antimesometrial cells now contain a few large granules, and the number of cells in which they are abundant has increased. In material fixed or mordanted for 1–2 days in osmic acid mixtures the antimesometrial region is differentiated from the mesometrial in that it appears much darker. This is due to the darkening of the ground

substance of the cells, although all the granules which stain with Sudan III are not blackened. This reaction to osmic acid persists to the eleventh day, although the number of granules decreases. It should be noted that corresponding to the plasma reaction with osmic acid there is a diffuse yellowish-orange staining of the cytoplasmic ground substance in the colloidal Sudan III preparations.

The hypertrophied cells around the mesometrial end of the lumen show a number of fair-sized granules in addition to the few minute granules which are found in the remainder of the zone.

Seven-day–21-hour stage.—The entire transitional area of epithelium is now a mass of nuclei, red blood cells, and lipoid granules. The mesometrial area has only the distal granules described above. However, a site from the same uterus, fixed in chilled solutions, shows in addition numerous basal spherules which are not visible in the specimen fixed at room temperature. Antimesometrially the number of nuclei has decreased. The primary decidual and implantation zones show the same lipoid picture as the preceding case. This is true also of the secondary zone, except that the innermost cells usually have fewer large granules. The mesometrial region has only a few minute granules. As in the case of the epithelium, the lipoid of the primary and secondary decidual zones is more abundant when chilled fixatives are used.

Eight-day–12-hour stage.—This implantation site was fixed at room temperature, and only a few of the granules have been retained. The whole of the secondary decidual zone shows the diffuse yellowish orange, or blackening, when osmic acid is used. The central half of the zone, which was richer in granular lipoid in the 7-day case, now stains more intensely than the other half. All the granules have not disappeared, however. Very few cells of the mesometrial region show any indication of lipoid.

Nine-day–12-hour stage.—The implantation zone has a lake of blood in its center, and the cells which surround it contain many large lipoid granules. Also a few nuclei surrounded by lipoid granules are found in the lake. The striking feature of the remainder of the zone is that a few lipoid granules lie in the unstained cytoplasm of the cells. The primary and secondary decidual zones show a yellowish-orange coloration under low magnifications, but it is impossible to find any granules. The vacuolated cytoplasm of the mesometrial cells lying in the meshwork of sinusoids also stains with Sudan III, but granules are rare.

Eleven-day stage.—There has been no change in the lipoid. The implantation zone still has granular lipoid, whereas the diffuse yellowish-orange staining of the secondary decidual zone and the vacuolated cells of the mesometrial region are as pronounced as in the previous case.

Twelve-day stage.—The antimesometrial decidua, which is now a decidua capsularis (Fig. 1, *H*), consists only of several layers of elongated cells. They still stain diffusely. The appearance of an intermediate zone, free of lipoid, is the only change found in the mesometrial region (see "Glycogen" section, pp. 222 ff.).

GLYCOGEN

Six-day stage.—Whereas in later stages the mesometrial decidua is especially characterized by intracellular glycogen, at this time glycogen is only found antimesometrially. There, it is present in approximately half of the cells of the primary decidual zone at the sides of the implantation chamber and an occasional cell beyond it (Fig. 13). It appears in the wall of vacuoles as discrete granules after fixation in formol alcohol.

The remainder of the stroma is free of glycogen. It has been impossible to demonstrate glycogen in the epithelium at any stage.

Six-day–9-hour stage.—Glycogen makes its appearance in the newly hypertrophied cells immediately adjacent to the transitional epithelium. This marks the beginning of what may be called the "glycogenic" area (Fig. 1, *C* and *D*).

Seven-day stage.—Very few cells in the primary zone stain with Best's carmine, but the glycogenic area has increased in extent.

Seven-day–8-hour stage.—Glycogen has disappeared from the antimesometrial region and does not reappear. The glycogenic area is 4–6 cells in width. Its relations may be seen in Figure 1, *D*, where it is indicated by hatching. The hypertrophied cells of this region are loaded with glycogen. The subsequent spread of the glycogenic area into the mesometrial decidua (Fig. 1, *E*) is indicated by the appearance of an occasional flake of intracellular glycogen.

Seven-day–12-hour stage.—In this case the glycogen has disappeared from the cells adjacent to the uterine lumen which accordingly resemble the cells of the primary decidual zone. They remain free of glycogen. As indicated in Figure 1, *D*, the capillaries lateral to the glycogenic area have already become prominent. In their vicinity the cells are now laying down glycogen so that the wings of the area are foreshadowed (Fig. 1, *E*).

Eight-day–5-hour stage.—In a continuation of the growth noted in the previous case, the capillaries of the glycogenic area have become sinusoidal, and some of them open into the blood space surrounding the Träger. The entire mesometrial decidua is larger. The enlarged cells, which are never binucleated, lie in groups separated by sinusoids and invested with reticulum fibers arranged in bundles. The inner edge of the glycogenic area is sharp; cells loaded with glycogen are bounded by cells free from it. Proceeding laterally the glycogenic area fades off gradually as the cells become smaller and the intracellular spaces larger. At the lateral extremities, and mesometrially, the cells are stellate, and only a few contain glycogen.

Nine-day–12-hour stage.—The sinusoids have enlarged and have become more abundant so that the glycogen-laden decidual cells appear as islands surrounded by endothelium. Their distribution is seen in Figure 1, *F* and *G*. They are the forerunners of the maternal blood channels of the definitive placenta, and at this time their distribution is largely confined to the glycogenic area. Decidual cells have been described in these sinusoids, but in most instances they are only slices of the irregular islands cut off in sectioning since they are surrounded by endothelium.

As in the earlier stage the specific characteristics of the area fade off at the periphery. With the spread of the area the mesometrial central area becomes smaller (Fig. 1, *F*, coarse hatching) as the cells at its periphery develop glycogen. Even at this stage there are scattered decidual cells in it which contain some glycogen.

Ten-day stage.—The glycogenic area with its sinusoids has pushed still farther mesometrially (Fig. 1, *G*). The decidual cells containing glycogen in the central area have increased in number but are still by far in the minority.

Eleven-day stage.—The development of the Träger and the enlargement of the gestation sac as well as the spread of the glycogenic area contribute to the reduction of the central area. The sinusoids have spread throughout the mesometrial region but are closer together in the decidua overlying the yolk sac (Fig. 1, *H*). This suggests resorption by the yolk sac.

At the narrowed antimesometrial extremity of the glycogenic area the elongated cells are vacuolated and contain much glycogen, except where they lie in contact with the embryonic tissue. Proceeding toward the implantation axis, where glycogen is much more abundant than in the previous case, typical decidual cells are encountered. The central area is now reduced in size, and fully one-half of its cells contain glycogen.

Glycogen-free endothelium lines the sinusoids where they are intact. Occasionally there is a free cell containing glycogen within them.

Twelve-day stage.—Previously the glycogenic area has been in contact with the Träger (ectoplacental cone of Duval). Now a narrow zone has appeared between them. This is due to the regressive transformation of the decidual cells which have elongate nuclei, vague boundaries, and little glycogen. Sinusoids are almost lacking.

The remainder of the maternal placenta has not changed fundamentally except for a decrease in glycogen. Another feature which may be mentioned is that in the center of the maternal placenta some of the endothelial cells have enlarged. In these sinusoids, and in the spaces of the fetal placenta containing maternal blood, there are a few free, glycogen-laden cells.

CYTOLOGICAL SUMMARY

The foregoing description clearly indicates that there are fundamental differences between the antimesometrial and the mesometrial decidual cells. First, the nucleus of the antimesometrial cell is relatively large and double in approximately one-third of the cells. One or 2 eccentrically placed nucleoli are present, and the chromatin is usually in large clumps near the periphery. The cytoplasm is decidedly granular, and a distinct cell membrane is present. Some of the granules contained in the cytoplasm may be lipoidal, and in the majority of the cells these granules fill much of the cytoplasm (Fig. 3). The more lipoid these cells contain, the more difficult it is to demonstrate the mitochondria which when seen are short, fine filaments with a few long threads and spheres (Figs. 10 and 11).

The mesometrial cell does not become so large as the antimesometrial cell. Whereas the antimesometrial cell is always readily isolated in fresh material, it becomes more difficult to isolate the mesometrial cell as the gestation age increases. The nucleus differs from that of the antimesometrial cell in that it is always single and is likely to shrink in fixation. Unless the lipoid and glycogen are preserved, the cytoplasm appears vacuolated. The lipoid is never abundant. On the other hand, large amounts of glycogen may be present. The mitochondria differ from those of the antimesometrial cells only in that they are more readily stained.

Mesometrially there is a third type of cell the origin of which has not been determined. This compares in size to the mesometrial decidual cells, and the nucleus has an indentation at one side and stains deeply. The cytoplasm is sparce. Mitochondria are perinuclear and tend to be granular in form.

DISCUSSION

The extensive antimesometrial decidual formation is to be correlated with three conditions: first, the glands are few and do not appear so active as they are in many mammals, nutrition for the embryo being provided by the stroma; second, as Duval (1897) suggested, the reaction may be associated with the peculiar development of the fetal membranes; and, third, it provides the decidua capsularis.

The reaction has been regarded as one of growth and degeneration which ultimately leads to the formation of a capsule for the embryo by Tafani (1886), Duval (1891), Jenkinson (1902), Burckhard (1901), Kolster (1903), and others in the mouse and rat. The cytolysis of decidual cells by fetal elements has been the explanation for the enlargement of the implantation chamber. In the rat this is not entirely true. The loss of cells plays only a minor role in the thinning of the wall. When one considers that the diameter of the gestation sac at 12 days is greater than that of the entire antimesometrial decidua at 9 days, it becomes apparent that the thinning of the wall is due to the increasing amount of surface to be covered. Such tissue loss as there is can be explained on the hypothesis of physical pressure being exerted upon these cells great enough to cause their death.

The decidual reaction, then, is a normal but exceedingly vigorous growth in which the few signs of degeneration can be accounted for on a mechanical basis. Thus, the formation of binucleated cells in the antimesometrial region may be regarded as a feature of the rapid growth; they do not become multinucleated, nor is there any fusion to form a symplasma as Burckhard (1901) noted in the mouse.

It has been inferred that the presence of the lipoid in the decidual cells is to provide a source of nutritive material for the embryo. Such a concept is supported by the dispersion and increase in lability of the lipoid at the start of the eighth day. At this time the yolk sac has been differentiated and, in addition, capillaries have opened into the implantation sac. These phenomena support the suggestion of Lee (1918), Brunschwig (1927), and others that the physiological yolk-sac placentation precedes the development of a definite placenta. From this point of view the preceding period of granular lipoid development must be regarded as a storage phase.

In so far as I have been able to determine, no one has described glycogen in the antimesometrial decidua, and Szendi (1933) denies its presence in this location. There can be no question, however, that it is present in the primary decidual zone in early implantation stages. The reason for its prompt disappearance is a question that remains unanswered.

The controversy over the origin of the cells of the glycogenic area in various mammals —Maximow (1897), Chipman (1902), Goldmann (1912), Gérard (1926), and others—can be definitely settled so far as the rat is concerned. In this animal all the stroma cells of the mesometrial region may contain glycogen. Also, it is quite apparent that the formation of decidual cells is not merely a deposition of glycogen. However, the general elaboration of glycogen, as well as decidual growth, are definitely correlated with the increase and enlargement of capillaries into sinusoids. The only explanation of the decidual glycogen and lipoid is that they are food reserves for the embryo.

To summarize, the rat uterus responds to the embryonic stimulation first in the formation of an antimesometrial decidua characterized by large amounts of intracellular lipoid and then in a mesometrial reaction involving an elaboration of intracellular glycogen before the cells enlarge. It is impossible in cytological preparations to differentiate the stroma cells of the two regions. Thus, it is a question whether we are dealing with two physiologically distinct cell types, which respond differently and at different times to the same stimulus, or whether differences in blood supply or some similar condition account for the difference in response of the two regions. It will appear below that these differences are inherent in the uterine stroma and are not due to differences in the effects of embryonic structures.

The Deciduoma Reaction

The deciduoma reaction, first observed by L. Loeb (1908), has been studied by various investigators, among whom may be mentioned Hammond (1917), Nielson (1921), and Brouha (1934) in the rabbit; Frank (1911), Corner and Warren (1919), and Long and Evans (1922) in the rat; Parkes (1929) in the mouse; and Krainz (1914) in the bitch. The majority of investigators have been satisfied to obtain the reaction, and it appears that none of them has been interested in the minutiae of the deciduoma. It has been shown, however, that there are various regional differentiations in normal pregnancy. The question accordingly arises: Is the deciduoma formation an unorganized tumerous reaction on the part of a sensitized stroma, or are there correlated regional differentiations comparable to those found in the normal decidual reaction? The answer is unambiguous, as will appear in the following pages.

TABLE 2*

DECIDUOMATA PRODUCED BY MECHANICAL STIMULATION

Rat No.	Gestation Age at Time of Stimulus		Total Gestation		No. Embryos in Normal Horn	No. of Deciduomata	No. of Stimuli	Nature of Stimulus
	Day	Hour	Day	Hour				
58	4	12	8	12	4	5	5	Cautery
62	2	14	6	2	5	3	5	Paraffin†
63	2	18	6	8	4	2	5	Thread†
64	3	11	6	11	3	2	5	Steel
66	4	.....	6	15	0	3	7	Steel
67	4	13	7	23	4	5	8	Glass
69	4	12	7	13	4	4	6	Steel
70	4	.....	7	.....	4	5	7	Steel
72	4	11	6	18	4	5	5	Glass
73	4	11	6	21	7	6	6	Glass
76	4	13	9	.....	0	6	6	Glass
81	4	13	12	1	4	7	9	Glass
83	4	12	10	15	4	4	7	Glass

* Rat No. 58 was the only multiparous animal used. In 5 cases deciduomata failed to develop when stimulation was applied before the third or after the seventh day. However, in 4 other cases the reaction failed although there were normal pregnancies in the normal horn. Of the deciduomata obtained 40 were used for study.

† Bits of thread or paraffin were inserted into the lumen through a minute incision at the tubal end of the horn.

MATERIAL AND METHODS

In order to obtain the normal hormonal balance of pregnancy the deciduomata were produced in the empty horns of unilateral pregnancies. Prior to the time of breeding, one Fallopian tube was tied and resected in part. In some cases this operation was done during pro-oestrus, but in only one case did the animal mate on the same day. The others did not mate until the next oestrus period.

In a preliminary series of experiments various methods for the production of deciduomata which have been described were tested (Table 2), and the thermocautery was also tried. However, all these methods produced trauma with some extravasation of blood. To avoid this an electrical stimulation method was devised which introduced fewer vari-

able factors and was used in a second group of animals (Table 3). The method consists of simultaneously bringing the two electrodes into contact with the mesometrial and antimesometrial surfaces of the empty uterine horn. There were definitely localized decidual reactions corresponding to the contact points. As many as 6 enlargements were obtained in a single horn. Twelve deciduomata thus obtained were selected for cytological study.

TABLE 3

DECIDUOMATA PRODUCED BY DIRECT-CURRENT STIMULATION

Rat No.	Gestation Age at Time of Stimulus		Total Gestation		No. Embryos in Normal Horn	No. of Deciduomata	No. of Stimulations
	Day	Hour	Day	Hour			
51	4	12	6	11	3	2	8
57	4	14	8	20	5	4	6
82	4	13	6	19	5	6	8
85	4	11	10	12	6	5	7
86	4	14	6	14	3	6	6
94	4	12	10	13	7	2	7
95	4	12	11	13	2	1	5

TABLE 4

DECIDUOMATA PRODUCED IN A PREGNANT HORN

Rat No.	Gestation Age at Time of Stimulus		Total Gestation		No. Embryos in Normal Horn	No. Embryos in Experimental Horn	No. of Deciduomata	No. of Stimulations	Nature of Stimulus
	Day	Hour	Day	Hour					
49*	4	15	9	12	2	5	2	4	Glass
53†	4	13	11	.....	6	3	2	8	Glass
54†	4	14	9	.....	4	2	4	4	Glass
79	4	13	9	22	4	2	3	4	Glass
80	4	13	8	13	1	0	1	6	Glass
89*	4	12	10	.....	0	2	1	4	Glass
90*	4	12	8	.....	7	2	4	5	Glass
91*	4	11	6	23	2	2	3	5	Glass

* The intact pregnant horn was stabbed.

† Successful transplants to the kidney of the removed portion of the horn.

In a third group of animals, during the fourth and early fifth days after copulation, a portion of the tubal end of the pregnant horn was removed for transplantation experiments (cf. p. 230). The remainder of the horn was then stabbed with a steel or glass needle and pregnancy allowed to continue. Fifteen deciduomata from this group were studied by cytological methods. The same methods of fixation and staining and fresh-tissue study were used as described in the first section of the paper.

OBSERVATIONS

Since the major features of development of the deciduomata so closely follow those found in implantation, it will suffice to point out the resemblances and differences between them. Also, since the electrically produced deciduomata follow the normal more closely than the others, they will be used as a basis. For convenience the period studied has been divided into daily intervals, and as nearly as possible the same features listed under the same headings. In all cases the age given represents the time elapsed since copulation so that the findings can be correlated as to time sequence with those of normal pregnancy.

SIXTH DAY

In normal implantation this is the period of embryonic attachment and the division of the antimesometrial region into its respective zones. Grossly a deciduoma may be mistaken for an implantation site of corresponding age since it is only slightly larger. When produced by mechanical stimulation, it is less sharply defined. The greater volume is due to an increase in oedema fluid in the muscular coat and in the inactive basal zone (Fig. 15). This is also true of the seventh-day cases.

Lumen.—The locus of stimulation may be regarded as analogous to the implantation. axis since the growth centers about it. There is a corresponding increase in the length of the lumen in this axis, and indications of its regional differentiation are present (cf. p. 214). In the cases of mechanical injury the instrument causes an antimesometrial displacement of the lumen, and usually a tongue of tissue protrudes into it. The tongue shows signs of degeneration whether it be purely epithelial or have a core of decidual cells. Small blood clots are present in most of the mechanically stimulated cases whereas in electrical stimulation only a few leucocytes may be found.

Antimesometrial region.—The development of the primary decidual zone along the implantation axis to the basal zone, and the subsequent separation of the two zones during the last part of the day by the development of the secondary decidual zone (cf. p. 218), occur as in normal implantation. That is to say, there is hypertrophy of the primary decidual cells with some mitosis, rapid hyperplasia, a hypertrophy, and the formation of binucleated cells in the secondary zone.

Capillaries.—Although the primary decidual zone does not have many capillaries extending into it, there are a few prominent ones just under the surface epithelium antimesometrial to the lumen. They correspond in position to the implantation zone which is otherwise not differentiated.

Lipoid.—The antimesometrial cells resemble the cells of normal implantation in all essentials, including the elaboration of lipoid and its distribution in the various zones. The epithelium usually is not so high and contains fewer basal spherules, whereas the epithelial cells of the tongue which is found in the mechanical stimulation are usually loaded with lipoid.

Glycogen.—Glycogen is demonstrable in the primary decidual-zone cells. As a rule it is more abundant than in normal implantation (cf. p. 221) since it is present in most of the cells of the zone. In mechanically stimulated deciduomata glycogen does not appear until the next day. The epithelium is glycogen-free as it is in all implantation specimens.

Mesometrial region.—There is as yet no reaction here as is the case in normal pregnancy.

SEVENTH DAY

In normal implantation the granular lipoid phase reaches its climax, and the mesometrial reaction is initiated. The deciduomata show similar changes.

Antimesometrial region.—The major growth of the antimesometrium is due to the hypertrophy of the cells in the secondary decidual zone. The hyperplasia of these cells has moderated greatly as in the normal condition, and the number of binucleated cells has increased. The basal zone has decreased in extent.

Capillaries.—The capillary meshwork at the border of the primary decidual zone is loosened (cf. p. 218), and its prominence causes this stage to compare favorably with a 9–10-day normal pregnancy. By the end of the day these capillaries have begun to join the prominent capillaries along the sides of the lumen to bring about the condition found normally late in the seventh day (cf. p. 218). They are not opened, however.

Lipoid.—The deposition of granular lipoid reaches a climax in extent early in the day. At this stage a cross-section at right angles to the implantation axis would give the same picture as that of normal implantation except that the capillary bed, rather than the implantation zone, would be at the center (cf. p. 220). On the whole, the deciduomata have slightly less lipoid than the normal in that fewer cells are heavily loaded with the larger granules. However, the arrangement of granules within the cell is the same (cf. p. 220 and Figs. 4 and 5). During the later part of the day the number of granules is decreased. Occasional cells in the mesometrial region contain a few granules.

Glycogen.—The glycogen distribution is not unlike that of the implantation sites. During the course of the day it decreases in the primary decidual zone and there is a gradual migration mesometrially so that by the end of the day the glycogenic area with its associated sinusoidal meshwork is well defined. This is slightly in advance of the condition found normally.

Mesometrial region.—The mesometrial differentiation starts in the seventh day and is characterized by the differentiation and enlargement of capillaries to form sinusoids about the mesometrial end of the lumen by the end of the day. At that time they are continuous with the capillaries of the antimesometrial region, forming an incomplete vascular ring about the lumen.

Reticulum.—In fresh-tissue studies the entire decidua appears to be more fibrous than in normal implantation. This is not due to the reticulum, for in stained sections its appearance in deciduomata is very simular to that seen in the normal decidua. The only difference is to be found in the absence of the implantation zone in the former (cf. p. 218).

EIGHTH DAY

A change in the nature of the lipoid and continued mesometrial development are characteristic of normal pregnancy. In the deciduomata the following development occurs:

Lumen.—The lumen is shortened and assumes a central position during the course of the day owing to the hypertrophy of the mesometrial region. In many cases its low cuboidal epithelium is incomplete.

Antimesometrial region.—The cells in the outer portions of the secondary decidual zone continue to enlarge.

Capillaries.—The peripheral portions of the capillary meshwork of the younger stages is farther dispersed whereas the vascular ring about the lumen becomes more complete. Only the presence of the epithelium differentiates it from the normal site. At this time it fully occupies an area corresponding to that of the implantation zone, and only a few decidual cells are dispersed among the closely packed capillaries.

Lipoid.—The lipoid of the antimesometrium changes from the granular to the dispersed condition. The decrease in the number of granules is relatively the same as in normal implantation (cf. p. 221).

Glycogen.—The glycogenic area as well as the sinusoidal area develop rapidly to form the winglike arrangement noted in the normal implantation (cf. p. 222). There is a loss of glycogen in the cells bordering the lumen, and the central area of the mesometrium becomes clearly differentiated.

NINTH DAY

Uniform size and distribution of the antimesometrial decidual cells are attained, and early maternal placental formation continues as normal implantation proceeds. In the deciduomata from this time on considerable variation in growth is encountered. During the seventh and eighth days the development has been slightly in advance of implantation. At this time, particularly if a sufficient number of stabs has been made, the whole horn may be enlarged and only slight depressions in the mesometrial wall distinguish the separate areas of reaction.

Antimesometrial region.—The liver-like appearance of the antimesometrial wall (cf. p. 218) is attained by the middle of the day and is like normal implantation in all its features.

Lipoid.—The lipoid is generally dispersed, and only occasional cells contain granules. The larger mesometrial cells may have a few granules.

Glycogen.—The glycogenic area has developed in the direction described for normal implantation although it is slightly in advance of it. Thus the central area is correspondingly small (cf. p. 222).

Mesometrial region.—The cells of the mesometrial region exhibit the characters described for the corresponding normal cells and, like them, do not attain the size of the antimesometrial cells. In addition, cells with the deep-staining indented nuclei and sparse cytoplasm are also found. All three cell types are easily recognized in fresh tissue.

Sinusoids.—The distribution of the sinusoids in their relation to the lumen merits particular attention. It has been noted previously that the surface epithelium is incomplete. At this time it may be defective mesometrially, leaving areas where the endothelium borders the lumen. In places this, too, is lost, and blood is found in the lumen. In mechanically stimulated cases this does not appear to be due to the initial trauma. In the case of the electrically stimulated deciduomata the continuity of the surface epithelium was not broken at the time of the stimulation. That the sinusoids are open intra vitam in such cases has been shown by the intravenous injection of colloidal mercuric sulphide (Hille) into the blood stream. After such treatment blood containing the sulphide may be found in the lumen of electrically as well as mechanically stimulated deciduomata. This is strong evidence that the extravasation is a feature of early maternal placental development and is quite independent of trophoblastic activity. The blood of the placental sign of Long and Evans is then probably due entirely to maternal reactions.

The presence of leucocytes noted in the younger cases has persisted, and at this stage they are abundant in the mesometrial sinusoids.

TENTH DAY

In pregnancy the thinning of the antimesometrial wall and the continued development of the glycogenic area are the outstanding features.

Antimesometrial region.—The antimesometrial wall has increased in thickness in contrast to the normal where the implantation cavity is becoming spherical and enlarging (cf. p. 218).

Lipoid.—The lipoid content does not vary from that of the normal.

Glycogen.—The glycogenic area and its associated sinusoidal net have progressed rapidly and have a structure much the same as that found in the normal 11-day case (cf. p. 222). Thus, the climax is reached approximately a day sooner, and the deciduoma has slightly more glycogen than the early maternal placenta of pregnancy.

ELEVENTH DAY

In normal pregnancy the antimesometrial wall thins to a decidua capsularis, and the glycogenic area reaches the climax of its development.

Lumen.—In the deciduoma the lumen may have appeared between the antimesometrial region and the basal zone so that two lumina appear in cross-sections of the enlargement. Both cavities are usually filled with a coagulum of blood, and cellular elements with some evidence of leucocytosis.

Antimesometrial region.—Because there is no rapidly enlarging lumen the antimesometrial wall has retained its thickness. Capillaries are abundant, and there are small areas in the central half of the region in which the cells are reduced in size.

Lipoid.—The dispersed lipoid is still present.

Mesometrial region.—The sinusoids increase in number, but there is a sharp decrease in the amount of glycogen.

KIDNEY TRANSPLANTS

The portions of uterine wall which were removed from pregnant horns (cf. p. 226) were washed in normal salt solution and transplanted to the kidney. Two such transplants were successful and corresponded to 9- and 11-day pregnancies. The cells were of the antimesometrial type. Although subjected to rough treatment in transplanting, only localized developments occurred. Not only were some of the cells binucleated but they also showed the condition of the lipoid corresponding to the respective stage of development. The inactive portions were comparatively free of lipoid. The surface epithelium, however, showed the typical pregnancy picture (cf. p. 215).

DISCUSSION

From the foregoing it is obvious that the growth of the deciduoma closely simulates the normal development of the decidua. This is especially true of those produced by electrical stimulation. The general histological features—that is, cellular distribution, time sequence, and vascular pattern, including extravasation—follow normal implantation closely. Cytologically the cells are fundamentally the same in nuclear and cytoplasmic structure and in the stored lipoid and glycogen.

Mechanical stimulation introduces certain insignificant complications such as the displacement of the lumen and its protruding tongue of tissue. The major differences between deciduomata and normal implantation sites are: (1) the absence of a rapidly enlarging implantation cavity; (2) associated with this, a distinct implantation zone does not develop although it is indicated by capillary differentiation; (3) mesometrially the central area is usually smaller than in pregnancy; (4) the major growth and glycogen distribution reach their climax during the tenth day whereas it is not reached in the normal until the eleventh; and (5) the greater part of the surface epithelium is not lost.

The explanation of how the epithelium is removed and the decidual reaction initiated by the embryo has not, as yet, been found. Turner (1876) suggested that the embryo acted as a parasite and as such removed the surface epithelium and initiated the decidual reaction. The majority of the morphologists have accepted this theory in the interpretation of their observations. Selenka (1883) gave such an interpretation to the reaction in the mouse and was followed by Spee (1901) for the guinea pig, Lee (1903) for *Citellus*, and various other authors who have studied rodents.

This interpretation, however, is not conclusive. Hubrecht (1890), although he left the impression that the embryo destroyed the surface epithelium, was not certain of this fact. Sobotta (1903) in a footnote suggests that the epithelium is removed as a result of physical pressure. Ercolani (1879) had observed that there was a proliferation of the stroma tissue prior to the time of embryonic attachment. Hubrecht (1890) confirmed this phenomenon in the hedgehog and agreed with Ercolani in recognizing that such a growth was normal in many mammals.

Specifically, for the rat, considerable evidence militates against the theory of active invasion by the embryo:

1. Momentary electrical stimulation involving no mechanical injury produces the development of a decidua which histologically and cytologically closely simulates the series of changes seen in normal pregnancy. These observations make it quite clear that this development is to be regarded as a definite and organized growth of specific regions in the fulfilment of their normal physiological function and lead us to question Loeb's (1908) suggestion that the deciduoma is essentially a tumerous growth.

2. In addition to the general histological and cytological development there is a corresponding vascular differentiation which proceeds as in pregnancy even to an extravasation of blood. Our observations on this point substantiate and elucidate the findings of Evans (1928). In vitamin-E deficient rats he not only observed spontaneous deciduoma development but also the placental sign (placental bleeding) in pseudopregnant animals. More striking is his observation of the same phenomena in 4 per cent of normal pseudopregnant animals. It is evident not only that the cells of the uterus elaborate food reserves for the embryo but also that maternal blood is provided even though the embryo is not present.

3. The majority of the deciduomata described by previous workers have been produced in pseudopregnant animals. Since we have also obtained normal deciduoma development of $9\frac{1}{2}$ days in animals in which implantation in the normal horn had failed, we can rule out any possible hormonal influence of the embryo in the differentiation of the decidua.

4. The observation of Brouha (1928) and others, that the deciduoma reaction will not occur unless the stimulus is applied within certain time limits, has been confirmed, i.e., under conditions of proper hormonal balance.

Thus, it is evident that the embryo is not essential for the development of the decidual reaction and that the stimulus need not be specific. The exact nature of the stimulus normally provided by the embryo must be left to future investigation.

Furthermore, once the reaction is started, what are the factors which direct and regulate its development? A partial answer to this question is found in the appareance of a deciduoma reaction in transplanted uterine stroma. The normal location, vascularization, and innervation are not necessary as has been shown by the transplantation experiments of L. Loeb (1908) and our own (cf. p. 230). From the evidence thus far obtained it

appears that we are dealing with an organ of specific potencies and that the inherent nature of the sensitized uterus merely requires some slight stimulus, or irritation, to initiate activity which continues for at least the first half of pregnancy when the proper hormonal environment is provided.

SUMMARY

1. In normal pregnancy the decidual response of the mucous membrane is different in the mesometrial and antimesometrial regions: (*a*) It appears first antimesometrially on the sixth day after insemination. (*b*) Here the stroma cells undergo hypertrophy and hyperplasia and accumulate labile intracellular lipoid. Binucleated cells are common and characteristic of this region. (*c*) The mesometrial reaction appears on the eighth day. The enlarged cells are loaded with glycogen, and the definitely localized dilation in the capillary bed is followed by some extravasation of blood.

2. Deciduomata were produced in most instances in the sterile horns of unilateral pregnancies. By electrical stimulation under conditions of proper hormonal balance deciduomata are produced which closely resemble the normal decidua, both in general form and in the vascular patterns. Extravasation occurs at a corresponding stage although no vessels were traumatized at the time of stimulation as they are by the methods of mechanical stimulation used hitherto. Both mechanical and electrical stimulation result in the development of characteristic antimesometrial and mesometrial decidual cells which elaborate lipoid and glycogen in the typical fashion.

3. Antimesometrial deciduae with characteristic cells were obtained in bits of uterus transplanted to the kidney. This indicates that the specific reactions are inherent in the stroma cells and are not due to their position and relations in the uterus.

Since the completion of this manuscript the paper of Selye and McKeown (1935) has come to my attention. Although the paper is devoted chiefly to a consideration of the hormones involved in placental formation and the structure of the "metrial gland," there are certain features bearing on our problem. On a histological basis these authors substantiate our findings (1935) of a differentiation of antimesometrial and mesometrial regions in deciduoma development. Their conclusion that the maternal blood vessels are opened without the activity of fetal ferments is fundamentally correct, but no adequate evidence for it is presented. After electrical stimulation and good fixation the blood sinus Selye and McKeown speak of proves to be the uterine lumen. In our material the subepithelial region (cf p. 229) is filled with sinusoidal vessels whose endothelium is intact, and the extravasation of blood occurs only in those regions in which the surface epithelium of the lumen is lacking; there is no indication of extravasation into the decidual tissue.

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PLATE I

Camera lucida drawings of cells fixed in Zenker-Bensley, imbedded in gelatin, cut after freezing, and stained for lipoid with Sudan III. ×1440.

FIG. 2.—Decidual cells of the primary decidual zone of a 6-day–4-hour pregnancy.

FIG. 3.—Cells of the secondary decidual zone of a 7-day pregnancy.

FIG. 4.—Cells of a secondary decidual zone of a deciduoma corresponding to a 7-day pregnancy.

FIG. 5.—Cells from the secondary decidual zone of a deciduoma corresponding to an 8-day pregnancy.

FIG. 6.—Epithelial cells of the antimesometrial area of a 6-day–4-hour pregnancy. These cells, as well as those shown in Figures 7, 8, and 9, are all from the same section.

FIG. 7.—Epithelium of the transitional area showing in part the transition to the implantation area.

FIG. 8.—Mesometrial epithelium.

FIG. 9.—Implantation epithelium.

PLATE II

Camera lucida drawings showing mitochondria of the antimesometrial cells. Fixed in Zenker-Bensley and stained with aniline acid fuchsin followed by Mallory's connective-tissue stain. Four μ thick. ×1440.

FIG. 10.—Cells of the secondary decidual zone of a 6-day–15-hour stage.

FIG. 11.—Cells of the primary decidual zone of a 6-day–2-hour stage (gelatin-imbedding and frozen-section technique).

PLATE III

FIG. 12.—A section of mesometrial decidua of an 8-day pregnancy. The cells bordering the lumen are glycogen-free as are the endothelial cells of the sinusoids. Fixed in formol-absolute alcohol and stained with Best's carmine. Initial ×115.

FIG. 13.—A cross-section of a 6-day implantation site. Only the implantation chamber with the surrounding primary decidual zone is shown. The differentiation in the surface epithelium is apparent. The cells of the primary decidual zone are to be regarded as decidual cells with a number of them showing glycogen in their cytoplasm. Fixed in formol-absolute alcohol and stained with Best's carmine. Initial ×65.

FIG. 14.—Cross-section through the implantation site of a 5-day–22-hour pregnancy. The distribution of the glands indicates the differentiation of a mesometrial and antimesometrial region. Furthermore, the compactness of the predecidual cells about the antimesometrial two-thirds of the lumen is the first indication of the primary decidual zone. Lipoid is only present in the epithelium of the lumen and the glands. Fixed in Zenker-Bensley and stained with Sudan III. ×25.

PLATE IV

FIG. 15.—Cross-section of a deciduoma corresponding to a 6-day–21-hour pregnancy. The larger size of the growth, when compared to the normal, is seen to be due to the oedematous condition of the basal zone. The lumen has been pushed mesometrially, and the lighter region extending antimesometrially from it is the primary decidual zone. Note that but few capillaries extend into it although they are numerous in the lipoid-laden secondary decidual zone. There is a slight reaction of the mesometrial region. Fixed in Zenker-Bensley and stained with Sudan III. Initial ×20.

FIG. 16.—A cross-section of a deciduoma corresponding to a 9-day–12-hour pregnancy. The section passes through the deciduoma slightly to one side of the center of reaction; thus the central area of the mesometrial region is not shown. However, the cells bordering the mesometrial surface of the lumen are glycogen-free. The quantity of glycogen is comparable to that of an 11-day pregnancy as are the development and distribution of the sinusoids. The antimesometrial decidua is seen to be quite uniform. No myometrium is included. Fixed in formol-absolute alcohol and stained with Best's carmine. ×25.

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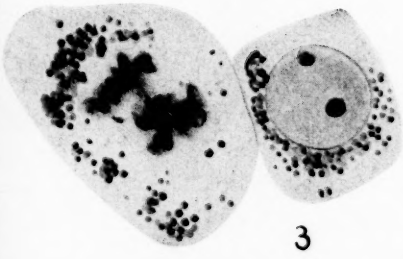

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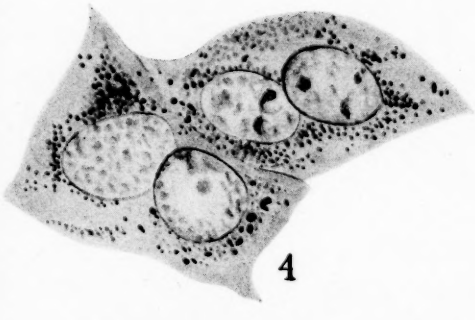

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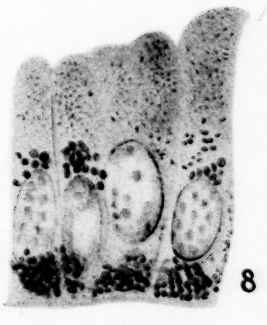

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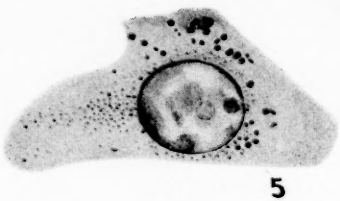

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PLATE I

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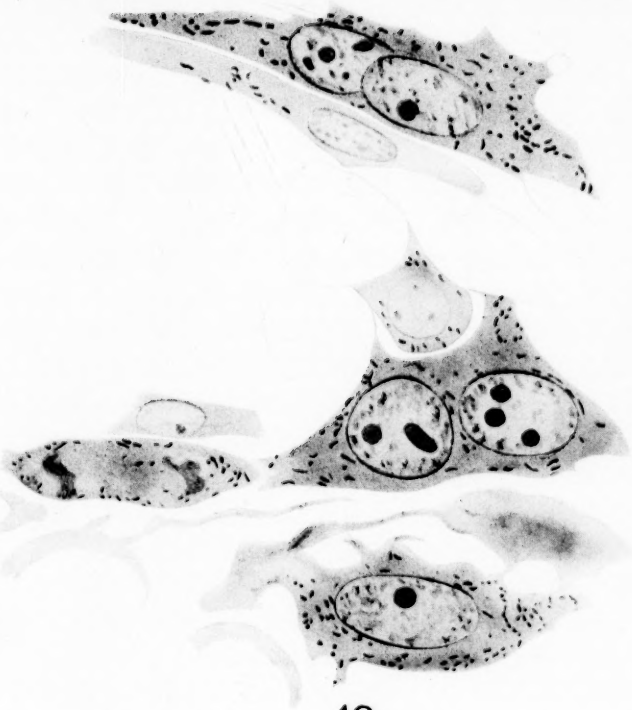

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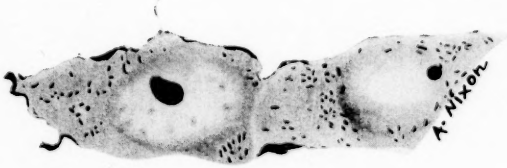

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PLATE II

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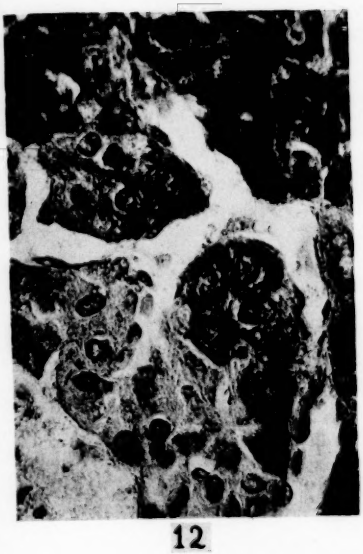
PLATE III



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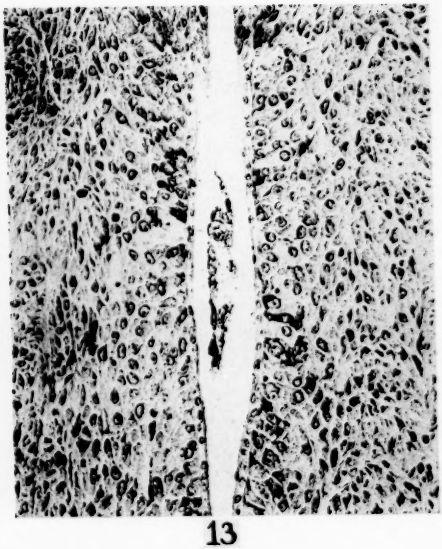

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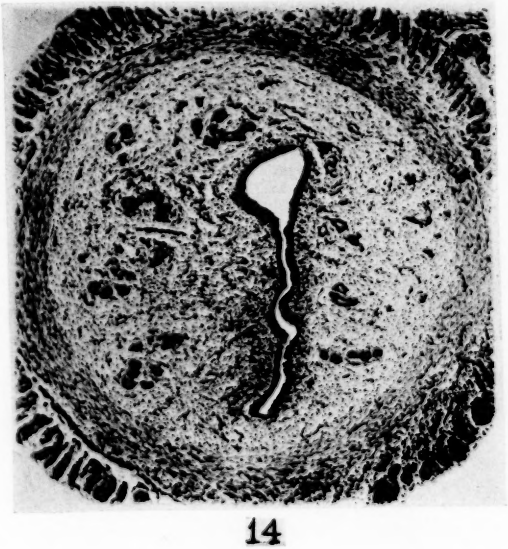

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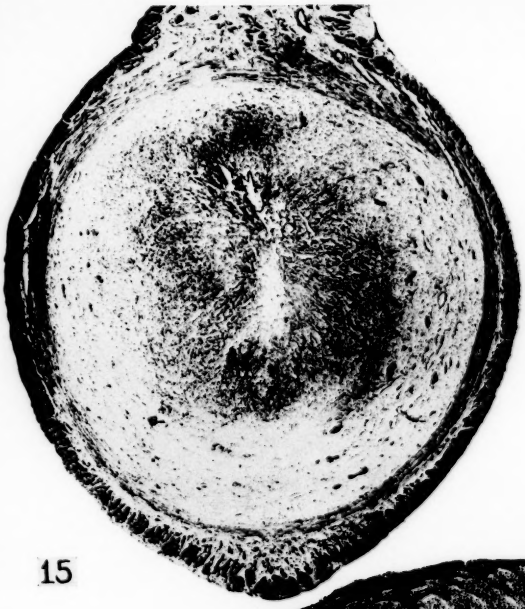
PLATE IV



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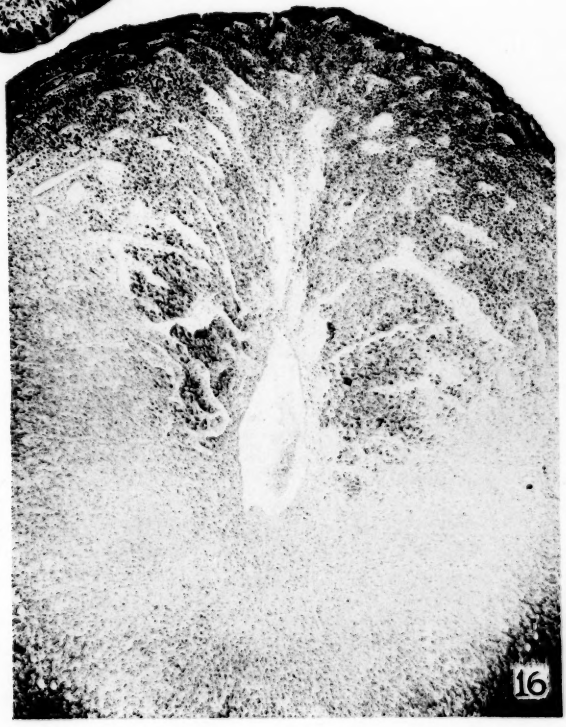

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PHYSIOLOGICAL STUDIES ON RESPIRATORY METABOLISM[1]

(Two figures)

PAUL R. ORR
University of Pennsylvania

FOR a variety of genetic and physiological experiments *Drosophila melanogaster* has been widely used as experimental material. During the development of the insect striking structural and physiological changes occur. Many of these changes have been reported in the literature. In a previous paper by Bodine and Orr (1925) it was shown that the rates of oxygen consumption and carbon dioxide output of pupae (*Drosophila*) follow definite U-shaped curves. Weinland (1905), Tangl (1909), Krogh (1914), Bodine and Orr (1925), Fink (1925), Clare (1925), Taylor (1927), and Frew (1929) found that the rates of oxygen consumption or of carbon dioxide output of the pupae studied give a U-shaped curve; the rates are high at first, decrease to a minimum, and then rise to a maximum value..

It has seemed advisable to continue these physiological studies with measurements on adult flies. In addition, it was thought to be of some interest to determine whether mutant stocks would differ from the wild type in the matter of oxygen utilization. The work here reported was undertaken to determine the rates of oxygen consumption of two different stocks or types of adult animals, the "wild" and the "vestigial" types, under conditions of complete absence of food. The relationship between the mutants and the wilds was further indicated by a study of a comparison of the oxygen consumption of these types when the sexes were kept separate under conditions of feeding and starvation, as well as when the sexes were combined under conditions of feeding. A study was also made taking into account the "hybrid" (wild × vestigial).

Through the present investigation interest has been focused on the respiratory behavior of *Drosophila* as individuals and not on the absolute amounts of oxygen utilized. The greater part of the work is based on a comparative study of relative rates of oxygen consumption by groups of flies.

MATERIAL AND METHODS

Two different stocks or types of *D. melanogaster*, "wild" and "vestigial," were used in these experiments. These types were obtained from Dr. R. L. King and had been bred continuously by him for many generations.

The flies were cultured in the laboratory at room temperature in half-pint milk bottles and fed a banana-agar-yeast mixture often used by geneticists and others working with these animals (Bridges, 1921).

The experimental animals were kept in half-pint milk bottles in an electric incubator at 25° C. Small pieces of filter paper were put in the culture bottles so that the larvae

[1] The author wishes to express his appreciation of the kindness of Dr. J. H. Bodine, under whose direction the investigation was conducted. Thanks are also due Dr. R. L. King for his helpful suggestions throughout the progress of the work.

could crawl up on them and pupate. The filter paper with the attached pupae was removed at regular intervals and the older pupae, as judged by apparent development and coloration, were removed so as to obtain pupae of approximately the same age. These pupae were then put into half-pint milk bottles containing small pieces of moistened filter paper to prevent their drying. The bottles were marked, stoppered with a small cotton plug, and kept in an incubator at 25° C.

By isolating experimental animals in this way, by cleaning, washing, sterilizing in 95 per cent alcohol, and again washing the pupae, it is possible to make certain that the

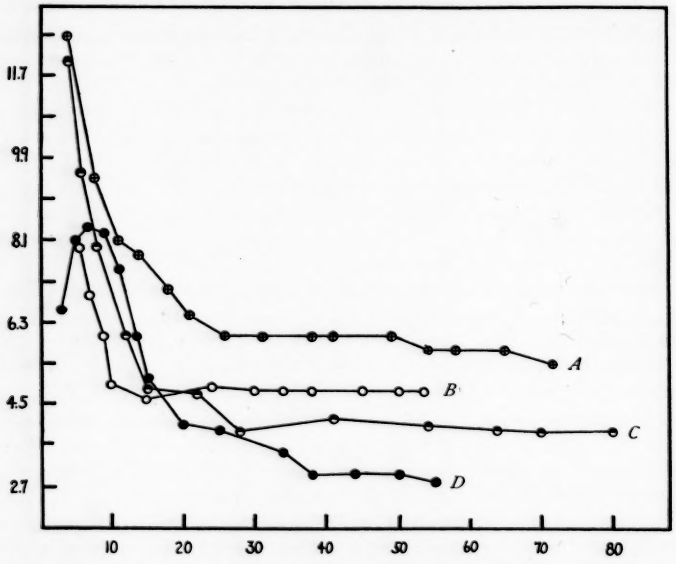

FIG. 1.—Shows the trend of oxygen consumption of wild- and vestigial-type *Drosophila* during starvation. Temperature, 25° C. *A*, vestigial females; *B*, vestigial males; *C*, wild females; *D*, wild males. Ordinate represents manometer units in millimeters; abscissa, time in hours after emergence.

emerging imagos will not only be of the same age but that they will be prevented from securing food of any kind during the period of experimentation.

By observing the time at which the flies emerged, flies of known age, in hours, were isolated. When the majority of flies had emerged, they were transferred to another half-pint milk bottle and then placed in an incubator at 25° C. Only sterilized bottles were used in this process. Trays of wet sand were placed in the bottom of the incubator to prevent any significant desiccation of the flies.

Flies used for a comparison between sexes were obtained from the same culture bottle, thus insuring the same environmental conditions. Those used in the sex-association experiments were obtained in the same way. Flies used for a comparison between the "types" were collected from cultures which contained banana mixture from the same lot, and all were kept under identical environmental conditions.

For oxygen determinations a modified Krogh (1916) micro-respiration apparatus was used. The manometers were the type improved by Bodine and Orr (1925). For the methods of using the apparatus, derivation of formula, etc., the reader is referred to Krogh's (1916) complete descriptions.

The experimental data reported in this paper were secured by two slightly different uses of the Krogh manometer. In one set of experiments the usual technique was employed. The flies were placed over NaOH in one of the two bulbs of the manometer. The data for Figures 1 and 2 are those obtained by using lots of ten flies at one time and taking readings throughout the experiment under conditions of complete absence of food

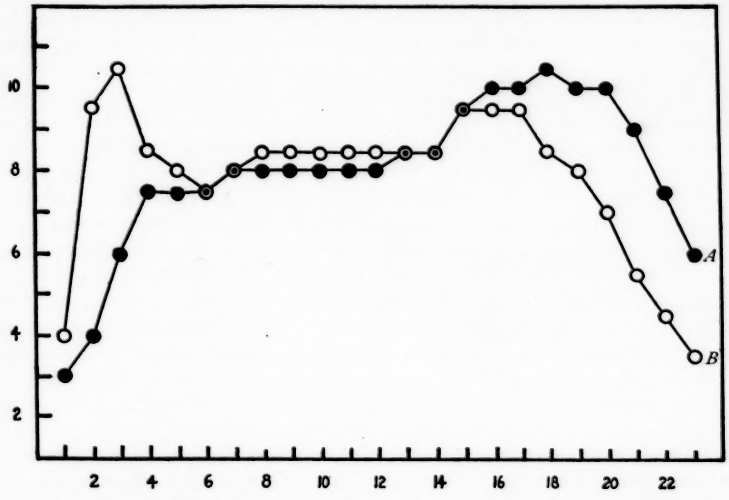

FIG. 2.—Shows the trend of oxygen consumption of wild- and vestigial-type *Drosophila* during starvation. Fifth-day pupae were placed in a manometer and their rate of oxygen consumption was taken hourly until several hours after the emergence of the flies. Temperature, 25° C. *A*, wild type; *B*, vestigial type. Ordinate represents manometer units in millimeters; abscissa, time in hours.

The flies in all cases had been living under identical environmental conditions and were of the same temporal age. In running through any series the same manometer was used, so that any possible variation in manometer volume would not complicate the experimental results.

In another set of experiments (Tables 1 and 2) the manometers were used in a somewhat different manner. Groups of flies were balanced against each other in the same manometer. For example, in the comparison between males of the wild type with males of the vestigial type, equal numbers of the two types were placed over NaOH in each of the bulbs of the manometer. The flies having the higher rate of oxygen consumption caused the kerosene of the manometer to move in their direction. This method was employed to indicate the difference in rates of oxygen consumption between the stocks and the sexes, and was also used to demonstrate the effect of feeding. Vestigial females and wild females, vestigial males and wild males, were studied under starvation conditions.

The same comparison was made under feeding conditions. Equal numbers of males and females of the vestigial types were compared with equal numbers of males and females of the wild type. The vestigial females were compared with the hybrid (vestigial × wild) females; the males, also, of these two types were used. Wild females were compared with hybrid females and, likewise, the wild males with the hybrid males.

OXYGEN CONSUMPTION

As pointed out above, the oxygen consumption and the carbon dioxide production for the pupae of *Drosophila* follow a U-shaped curve (Bodine and Orr, 1925). Figure 1, selected from many experiments, shows the oxygen consumption rates for the various types of adult flies in the complete absence of food. From an examination of curves *A*, *B*, and *C* it is evident that the adult has a high initial rate followed by a decrease during the first few hours, followed by a leveling-off to a constant rate. Curve *D*, showing a rise in rate of oxygen consumption during the first few hours of adult life in the wild males, suggests that the increase during the last two days of the pupal life continues after the emergence of the imago from the pupal state. To test this idea, fifth-day pupae were placed in a manometer and their rates of oxygen consumption were taken hourly until several hours after emergence of the flies. Figure 2 shows the results of such an experiment for wild and vestigial types. An examination of these curves points to the fact that the high rate of oxygen consumption of the last two days of pupal life is projected for a short time into the adult phase and that there is a characteristic subsequent decrease in the rate of oxygen consumption as pointed out in Figure 1. On the basis of curve *D* and Figure 2 it may be expected that curves *A*, *B*, and *C* would be similar to curve *D*—with respect to the initial rise in oxygen consumption immediately after hatching—if the adults had been studied just after emergence. The shape of the curves in Figure 2 is undoubtedly flattened out—the plateau between 4 and 17 hours—because of the fact that the pupae were not all in the same stages of development, although of approximately the same temporal age, so that some of the flies emerged before others. It was impossible under the conditions of the experiment to tell the exact time at which the flies emerged. In spite of this condition, the relation, in oxygen utilization, between the pupal and the adult stages is indicated.

Northrop (1926) measured the carbon dioxide production of *Drosophila* cultures. The curve in which the carbon dioxide production per 24 hours per 100 individuals is plotted against the time in days

confirms the results of Fink (1925), in that it shows a rapid increase during the larval period followed by a decrease in the pupal period. Since the curve is per individual and not per unit of weight, the rise is sharper than in the CO_2 per unit weight curve as it includes the increase in size of the larvae. It will be noted that the curve of CO_2 production of the imagos is practically constant throughout life.

The method used by Northrop for the measurement of CO_2 production, referred to above, did not permit the detection of well-known variations from time to time during the metamorphosis of insect pupae. Bodine and Orr (1925) have published results showing a definite U-shaped curve for the rate of CO_2 production and the O_2 consumption in *Drosophila* pupae. This was not detected by Northrop. The present work demonstrates the relation between late pupal and early adult oxygen utilization.

Pearce (1936), reporting on the oxygen consumption of excised liver, kidney, and resting ventricular cardiac muscle of a genetically pure strain of mice at two age periods ([*a*]

4 to 9 weeks and [*b*] 50 to 60 weeks), finds that the oxygen consumption of all three tissues in the older group is significantly less than in the younger group. In the same paper Pearce has reviewed the literature dealing with the changes in respiratory metabolism of various animals. It is clear that in all the animals studied —e.g., pigeon, fowl, man, pig, calf, rat, and dog—the basal metabolism declines, not necessarily at a constant rate, from a high peak of early youth to a lower level during adult life and old age.

STOCK DIFFERENCES

Previous work (Bodine and Orr, 1925) on the oxygen consumption and carbon dioxide production in pupae of *Drosophila* has demonstrated that a difference exists between the wild and vestigial types. Data calculated from Table II (Bodine and Orr, 1925) show differences in oxygen consumption (mm.^{3} per min. per 10 pupae) for the first two days to be 0.04466 mm.^{3} and 0.00566 mm.^{3}, respectively, with the wilds having the higher rate than the vestigials. On the third day the vestigials have a higher rate of oxygen consumption than the wilds, the difference being 0.000845 mm.^{3}; as the flies become older the difference is increasingly noticeable—0.02345 mm.^{3} for the fourth day and 0.0485 mm.^{3} for the fifth day. From Table II of Bodine and Orr (1925) it is also possible to calculate the differences that exist between the rates of oxygen consumption for the wild and the vestigial pupae on the basis of mg. O_2 per mg. body weight per min. When this is done, the wilds are found to have a higher rate than the vestigials for the first three days, the figures being 0.00001085 mg., 0.0000031 mg., and 0.00000065 mg. The vestigials have a higher rate than the wilds on the fourth and fifth days; the figures for these differences in mg. O_2 per mg. body weight per min. turn out to be 0.00000244 mg. and 0.00000713 mg. The foregoing data show conclusively that at the end of the pupal stage the vestigials have a higher rate of oxygen consumption than the wilds—regardless of whether or not body weights are taken into consideration.

Since late pupae of the vestigial type have a higher rate of oxygen consumption than late pupae of the wild type, as shown above, it might be expected that this difference would be continued into adult life. An examination of Table 1 points to evidence for the validity of this assumption.

Other stock differences between vestigial, wild, and hybrid (wild × vestigial) types of flies are also indicated in Table 1. When the wild type and the vestigial type females were compared in the complete absence of food, the vestigial-type flies were found to have the higher oxygen consumption rate; the same applies for the males. The length of life of the two stocks does not seem to be directly proportional to their rates of oxygen consumption since the vestigial-type female flies have the same duration of life in complete absence of food as do the wild-type females under the same condition of non-feeding; this fact also holds true for the males. For data on the duration of life of *D. melanogaster* in the complete absence of food see Pearl and Parker (1924).

Table 1 also lists experiments in which vestigial females were compared with wild females, vestigial males with wild males, and equal numbers of males and females of the vestigial type with the same number of wild-type flies (i.e., the sexes were combined in this experiment). The results showed that in all of these experiments the vestigial-type flies had the higher rate of oxygen consumption—under feeding conditions. As pointed out above, the late vestigial-type pupae have a higher rate of oxygen consumption than the wild-type pupae. In the adult stage this superiority is maintained: Vestigial flies have a higher rate of oxygen consumption than do the wild-type flies.

In a comparative study between vestigials and hybrids (wild × vestigial) under conditions of full feeding, it was found (Table 1) that for 18 hours at least after the emergence of the imagos the vestigials have a higher rate of oxygen consumption than the hybrids, for the same sex.

The results of the measurements of wilds compared with hybrids showed that the wilds have a higher rate than the hybrids; this is true for the females as well as the males.

An inspection of Table 1 furnishes evidence to show that the oxygen consumption of the three types studied follows this order: vestigials greater than the wilds and wilds greater than the hybrids when the flies are kept under as nearly identical conditions as possible.

TABLE 1

STOCK DIFFERENCES IN O_2 CONSUMPTION OF *Drosophila* ADULTS—TYPICAL EXPERIMENTS—GROUP OF 10 FLIES IN BOTH MANOMETER BULBS—25° C.

Age in Hours at Beginning of Experiment	Duration of Experiment in Hours	Manometer Unit (Mm.) Differences	Stock Advantage	
21	8	16.5	Vestigial ♀ > wild ♀	unfed
55	7	12.0	Vestigial ♀ > wild ♀	unfed
29	9	12.5	Vestigial ♂ > wild ♂	unfed
59	9	6.5	Vestigial ♂ > wild ♂	unfed
7	13	11.5	Vestigial ♀ > wild ♀	fed
6	10	10.0	Vestigial ♂ > wild ♂	fed
8	10	18.0	Vestigial ♂+♀ > wild ♂+♀	fed
7	14	16.0	Vestigial ♀ > hybrid ♀	fed
8	8	6.0	Vestigial ♂ > hybrid ♂	fed
6	9	10.0	Wild ♀ > hybrid ♀	fed
6	14	6.0	Wild ♂ > hybrid ♂	fed

The same results were found in experiments performed in a different manner. Females of wild, vestigial, and hybrid types were measured separately—i.e., the flies of each type were placed in only one of the bulbs of the manometer. The amounts of oxygen consumed were read directly, in manometer units. At the beginning of these experiments the flies of the three types were of the same age and the duration of each experiment was the same. The same manometer was used. The results of typical experiments are as follows: the oxygen-consumption difference in manometer units showed the vestigials to be greater than the hybrids by 15 units; the vesitigials greater than the wilds by 9.5; the wilds greater than the hybrids by 5.

In studies dealing with respiratory metabolism phenomena, one should consider other factors besides the respiratory gases involved. There are undoubtedly size, weight, and "activity" differences between the wild and the vestigial types of flies. The significance of the relation of body weight to metabolism in *Drosophila* can be appreciated by a review of Bodine and Orr's (1925) paper and Clare's (1925) paper. The pupal weights taken from the former paper show that these weights decrease from the first day of pupal life to the last day of that stage. The vestigials are consistently heavier than the wilds. As pointed out above, under the discussion of stock differences, there is a shift in the stocks

with regard to which type predominates in consuming the greater amount of oxygen. On the basis of mg. O_2 per min. per pupa, this shift from the wilds having the advantage over the vestigials to the vestigials having the advantage over the wilds occurs on the third day. On the basis of mg. O_2 per mg. body weight per min., the same change occurs on the fourth day. This shift cannot be due to relative body-weight changes, for throughout pupal life the vestigials are heavier than the wilds. The figures for the day just before the emergence of the fly show that the difference of oxygen consumption is 0.00000742 mg. O_2 per min. per pupa, the vestigials having the higher rate. The figure for oxygen consumption per mg. body weight per min., for the same day, is 0.00000713, the vestigials still having the higher rate. It can thus be readily seen that, for the day just before emergence of the fly, the vestigials have the higher rate of oxygen consumption—both per fly and per unit of body weight.

Complete figures are not available for the adult fly in regard to size, body weights, or "activity." Clare (1925) states:

> As applied to *Drosophila*, the practice of bringing metabolism into relation with pupal weight is warranted only in a most general way. It has been shown that a close approach to a knowledge of metabolism in *Drosophila* pupae cannot be made if sole dependence is placed on weight of respiring tissues as a guide.

SEX DIFFERENCES

It has been shown by at least two different methods that the female adult *Drosophila* flies have a higher oxygen-consumption rate than the males. Eddy (1931), in studying the rate of recovery from immersion in water, has shown that the *Drosophila* females have a slightly higher rate of metabolism than the males. Kucera (1934), using a modified Thunberg's microrespirometer, found that the females have a higher oxygen-consumption rate than the males, both per fly and per unit of body weight: 4.8 mm. difference in manometer reading per hour per fly or 0.000588 mg. of oxygen per mg. body weight per hour per fly, in favor of the females.

Data accumulated in the course of this work confirm—by still another method—the results mentioned above. Groups of ten males and ten females were placed in the two bulbs of the manometer and the movement of the kerosene recorded at the end of the experiment. The results of some of these experiments are given in Table 2. These figures show that the females of all types (wild, vestigial, and hybrid) have a higher oxygen-consumption rate than the males of the corresponding type at the time the measurements were made.

The data in Table 2 and the curves in Figure 1 indicate that the difference between the sexes, in rate of oxygen utilization, is not a constant value but fluctuates with the age of the flies. Kucera (1934) states that "on the next day after the flies emerged from the pupa cases their oxygen consumption was measured" and the females had a 52.2 per cent advantage (as shown by millimeters in manometer readings per hour per fly) over the males. This figure would undoubtedly vary, depending on the exact age of the flies at which determinations are made.

Results were obtained during the present study which suggest that, under certain conditions, toward the end of adult life there may be a shift from the condition of the females having the greater oxygen-utilization rate to a condition in which the males have the higher rate.

A survey of the literature on sex differences in metabolism of insects reveals consider-

able diversity in findings and theories by the various investigators. Krogh has suggested that the rate of metabolism is proportional to the amount of organized tissue present and capable of actively respiring. According to this assumption, the sex difference may be due to a greater proportion of organized tissue present in the female. Clare (1925) concludes from his studies that there is no significant sex difference in metabolic rate in *D. melanogaster* pupae. Taylor and Steinbach (1931) found that the male pupae of *Gallera mellonella* (bee moth) have the higher rates during approximately the first 150 hours (at 30° C.), while during the approximately 40 remaining hours of pupal development the females have rates considerably higher than the males. A significant sex difference exists between the maximum rates of the females and the maximum rates of the

TABLE 2

SEX DIFFERENCES IN O_2 CONSUMPTION OF *Drosophila* ADULTS—TYPICAL EXPERIMENTS—GROUP OF 10 FLIES IN BOTH MANOMETER BULBS—25° C.

Age in Hours at Beginning of Experiment	Duration of Experiment in Hours	Manometer Unit (Mm.) Differences	Sex Advantage
5	11	9.5	Wild ♀ > wild ♂ unfed
5	6	9.0	Vestigial ♀ > vestigial ♂ unfed
5	17	26.0	Hybrid ♀ > hybrid ♂ unfed
4	10	9.5	Wild ♀ > wild ♂ fed
34	7	12.5	Wild ♀ > wild ♂ fed
60	7	13.0	Wild ♀ > wild ♂ fed
90	6	11.0	Wild ♀ > wild ♂ fed
6	7	5.0	Vestigial ♀ > vestigial ♂ fed
35	7	12.5	Vestigial ♀ > vestigial ♂ fed
62	7	10.5	Vestigial ♀ > vestigial ♂ fed
90	7	8.5	Vestigial ♀ > vestigial ♂ fed

males, being higher for the females. These investigators are of the opinion that this is probably not a sex difference in basal metabolism but may be due to a difference in the relative amounts of actively respiring tissue present in the male and female pupae at corresponding periods of pupal development. Gowen (1931) has shown that the chromosomal constitution of various stocks of *Drosophila* is the determining factor for their metabolic rates. He found that "the type male produces 25 per cent more carbon dioxide than either the type females or triploid females." Park (1936), reporting on the oxygen consumption of the flour beetle, *Tribolium confusum* Duval, states that a statistical analysis of the data suggested that the weight difference between males and females was not the sole factor involved in explaining the higher oxygen consumption of the females.

As previously stated, the present study was based on groups of individuals and not on absolute amouts of oxygen consumed per body weight per unit of time. It is quite plain that, regardless of the conditions (starvation or full feeding), there is a difference between the sexes and types studied as evidenced by differences in rates of oxygen consumption.

SUMMARY

1. The oxygen consumption of vestigial females and males, as well as that of wild females and males, drops off rapidly under conditions of complete starvation.

2. The initial high oxygen consumption of the adults just after emergence corresponds to the high oxygen consumption at the end of the pupal period.

3. The differences in oxygen-consumption rates of the three types of flies studied follow this order: vestigials greater than the wilds and the wilds greater than the hybrids, when the flies are kept under as nearly identical conditions as possible.

4. Regardless of the condition of starvation or of full feeding, there is a difference between the sexes as evidenced by differences in their rates of oxygen consumption. The females have a higher rate than the males, at the times studied.

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